

Identification and Characterization of a Peptide That Specifically Binds the Human, Broadly Neutralizing Anti-Human Immunodeficiency Virus Type 1 Antibody b12

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Human monoclonal antibody (MAb) b12 recognizes a conformational epitope that overlaps the CD-4-binding site of the human immunodeficiency virus type 1 (HIV-1) envelope. MAb b12 neutralizes a broad range of HIV-1 primary isolates and protects against primary virus challenge in animal models. We report here the discovery and characterization of B2.1, a peptide that binds specifically to MAb b12. B2.1 was selected from a phage-displayed peptide library by using immunoglobulin G1 b12 as the selecting agent. The peptide is a homodimer whose activity depends on an intact disulfide bridge joining its polypeptide chains. Competition studies with gp120 indicate that B2.1 occupies the b12 antigen-binding site. The affinity of b12 for B2.1 depends on the form in which the peptide is presented; b12 binds best to the homodimer as a recombinant polypeptide fused to the phage coat. Originally, b12 was isolated from a phage-displayed Fab library constructed from the bone marrow of an HIV-1-infected donor. The B2.1 peptide is highly specific for b12 since it selected only phage bearing b12 Fab from this large and diverse antibody library.

Anti-human immunodeficiency virus type 1 (HIV-1) neutralizing antibodies (Abs) first appear months after the viremia that follows initial infection (1, 18, 27). This response, however, is highly type specific. Neutralizing Ab responses may broaden later in the infection (5, 24) but usually remain poor and occur sporadically in the majority of patients, including long-term-infected individuals (11, 23).

Only three broadly conserved neutralizing epitopes have been identified thus far on the viral envelope; they are defined by human monoclonal Abs (MAbs) b12, 2G12, and 2F5. MAb b12 binds to a discontinuous epitope that overlaps the CD4-binding site on gp120. MAb 2G12 recognizes a complex discontinuous epitope involving the C3-V4 region of gp120 and carbohydrate (34). MAb 2F5 binds to a linear epitope on the ectodomain of gp41 (8, 26, 32); however, the simplicity of this epitope is deceptive, since immunizations with recombinant influenza virus (25) or fusion proteins bearing this epitope (13, 17) have failed to produce significant 2F5-like neutralizing Ab responses, indicating that the native epitope on gp41 is more complex than the six-residue linear sequence. MAbs b12, 2G12, and 2F5 have shown *in vitro* neutralizing activity against a wide variety of primary isolates (7, 8, 12, 29, 33). Moreover, passive transfer of b12, 2F5, and 2G12 can provide sterile protection if adequate concentrations are achieved before HIV-1 exposure. Studies with 2F5, 2G12, and HIVIG showed that macaques were protected from intravenous (19) and vag-

inal (21) challenges with pathogenic SHIV_{89.6PD} (30). Passive immunization with IgG1 b12 protects hu-PBL-SCID mice from an HIV-1 primary-isolate challenge before and shortly after an intravenous viral challenge; (14) and macaques from a vaginal challenge with pathogenic R5 SHIV_{162P} (P. W. H. I. Parren, P. Marx, A. J. Hessel, A. Luckay, J. Harouse, C. Cheng-Mayer, J. P. Moore, and D. R. Burton, submitted for publication).

The success of these passive-immunization studies indicates an obvious goal in the development of a prophylactic vaccine: to elicit Abs having neutralizing activities similar to those of the currently known, broadly neutralizing MAbs (b12, 2G12, and 2F5). Yet, all of the recombinant envelope-based vaccine candidates tested so far in clinical trials have been unable to elicit significant neutralizing responses against HIV-1 primary isolates (9, 20, 22), even in cases in which b12, 2F5, and 2G12 bound well to the immunizing subunit antigen, indicating that their respective epitopes are antigenic on these forms of the envelope proteins. Furthermore, these neutralizing epitopes are not recognized to any significant degree during natural infection; instead, as mentioned above, serum Abs having only weak cross-neutralizing titers are typically produced. Of the large number of MAbs cloned from infected donors, b12, 2G12, and 2F5 are the only ones reported so far that neutralize a broad spectrum of primary HIV-1 isolates. Thus, although the epitopes known to mediate broad neutralization are present on recombinant envelope proteins and on envelope proteins produced during natural infection, they do not elicit significant neutralizing Ab responses against primary isolates.

The low apparent immunogenicity of these neutralizing epitopes on the envelope proteins may be circumvented if suitable small molecules mimicking them can be generated (i.e., molecules that bind tightly to the combining sites of the neutralizing MAbs) and then presented in such a form that

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Binding of HIV-1 gp120 to the nicotinic receptor

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We previously described a significant sequence homology between HIV-1 gp120 and the functional sites responsible for the specific binding of snake curare-mimetic neurotoxins and rabies virus glycoprotein to the nicotinic acetylcholine receptor. Here we report findings about the existence of a mechanism of functional molecular mimicry which could enable the binding of HIV-1 gp120 to nicotinic acetylcholine receptors in muscle cells and neurons.

Acetylcholine receptor; HIV-1; gp120; α -bungarotoxin; Mimicry

1. INTRODUCTION

In a previous paper [1] we reported a significant homology between the sequence, 164-174 [2], of HIV-1 gp120 and the putative active sites of snake curare-mimetic neurotoxins and rabies virus (RV) glycoprotein, which specifically bind to the nicotinic acetylcholine receptor (AChR). Curare-mimetic neurotoxins from Elapid snakes bind with high affinity to AChR and competitively block acetylcholine-induced membrane depolarization [3]. On the other hand the rabies virus binds to the muscle nicotinic receptor and this binding is inhibited by snake neurotoxins [4]. We consider the homology of gp120 with snake neurotoxins and RV glycoprotein to be of potential importance for HIV-1 infectivity in that it is centered around a region comprising highly conserved snake neurotoxin residues probably involved in receptor binding; moreover, in rabies virus glycoprotein, the same sequence corresponds to the site of rabies virus binding to AChR [5,6].

We proposed that nicotinic acetylcholine receptors can function as HIV-1 receptors in muscle cells and neurones, by virtue of mimicry of receptor-specific active sites of ligands by HIV-1 gp120. A similar mechanism is already suspected for rabies virus binding to muscle cells [6].

We found that recombinant gp120 from HIV-1 strain IIIB is able to inhibit the binding of the snake neurotoxin, α -bungarotoxin (α -Bgt), to the nicotinic acetylcholine receptor in the human rhabdomyosarcoma cell line, TE671. A 14-amino acid synthetic peptide (HG165-178: Asn-Ile-Ser-Thr-Ser-Ile-Arg-Gly-Lys-Val-Gln-Lys-Glu-Tyr), reproducing the sequence 165-

178 of gp120, homologous to snake neurotoxins and rabies virus glycoprotein, is also able, once conjugated to keyhole limpet hemocyanin (KLH), to inhibit the binding of α -Bgt to TE671 nicotinic acetylcholine receptor. Further, immunization of mice with the same gp120-derived peptide gave rise to antibodies efficiently cross-reacting with rabies virus glycoprotein and α -Bgt.

2. MATERIALS AND METHODS

2.1. Cell culture

The human cell line, TE671, was obtained from the American Type Culture Collection. Cells were grown to confluence at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum.

2.2. Iodination of α -bungarotoxin

α -Bgt was obtained from Sigma (St. Louis, MO, USA) and labelled with 125 I as described [7]; specific activity was $2-3 \times 10^{17}$ cpm/mol.

2.3. [125 I] α -Bgt binding to TE671 cells and inhibition by HIV-1 gp120

For binding experiments, cells were harvested mechanically with a rubber policeman and centrifuged at $450 \times g$ for 15 min; pellets were resuspended in phosphate buffered saline (PBS), pH 7.5, to a density of 10^7 cells/ml; 10^6 cells were incubated with 50 μ l of serial dilutions of HIV-1 gp120 (IIIB strain, Neosystem Laboratoire, Strasbourg, France) for 3 h under gentle stirring. 50 μ l of [125 I] α -Bgt (10^5 cpm) were then added and the cells incubated for a further 45 min. Binding was stopped by the addition of 1 ml ice-cold PBS containing 1 mg/ml BSA and samples were then centrifuged at $450 \times g$ for 15 min at 4°C. The cell pellets were washed twice as above and counted in a γ -counter (Minimaxi 500, Packard Instruments Co., Downers Grove, IL). Maximum binding was obtained by replacing inhibitors with assay buffer. Non-specific binding was determined in the presence of 7.5×10^{-6} M unlabelled α -Bgt. [125 I] α -Bgt binding in the presence of 1 mM nicotine and of 25 mM acetylcholine/0.25 mM neostigmine was also measured for additional controls.

2.4. Peptide synthesis

Solid phase synthesis was carried out with a model 430A automatic synthesizer (Applied Biosystems, Foster City, CA) employing F-moc chemistry. The peptide sequence was checked by a gas-phase microsc-

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WHY WOULD THIS BE USEFUL FOR TREATING HIV/RETROVIRAL INFECTION?

quencer (Model 470A, Applied Biosystem). HG165-178 was conjugated with KLH by glutaraldehyde, about 300 mol of peptide were bound per mol of KLH. The conjugated peptide (cHG) was used for inhibition experiments and for immunization of mice.

2.5. Inhibition of [125 I] α -Bgt binding to TE671 AChR by HG165-178 KLH conjugated peptide

5×10^5 cells in 100 μ l of PBS were incubated with 50 μ l of serial dilutions of peptides for 2 h. [125 I] α -Bgt (10^5 cpm) were then added and incubated for 45 min. The cells were washed and radioactivity counted as described above. Maximum binding was determined by replacing inhibitors with assay buffer. KLH and an uncorrelated 14-amino acid peptide conjugated to KLH (SI) were used under the same conditions to check non-specific inhibition.

2.6. Antithodies

Balb/c mice were injected intraperitoneally with 250 μ g of HG165-178 KLH-conjugated peptide in complete Freund's adjuvant (CFA) (day 1). The mice were boosted as above using incomplete Freund's adjuvant at days 15 and 36. At day 57 the mice were injected intravenously with 100 μ g conjugate in saline. After 3 days serum from immunized animals was collected for the experiments (polyclonal antibodies).

2.7. ELISA

Anti-HG165-178 mouse antiserum was tested on three different antigens. 96-well EIA plates were coated with HIV-1 gp120, RV glycoprotein or α -Bgt in 50 mM ammonium carbonate buffer, pH 9.5, for 18 h at 4°C. The plates were then washed and quenched with 3% bovine serum albumin, washed again and incubated with serial dilutions of antiserum for 3 h at 37°C. Binding was detected by horseradish peroxidase-conjugated anti-mouse IgG.

3. RESULTS AND DISCUSSION

A significant homology is present between the sequence, 164–174, of HIV-1 gp120 and the active sites responsible for the binding of snake neurotoxins and rabies virus glycoprotein to muscle AChR (Fig. 1).

The human rhabdomyosarcoma cell line, TE671, is known to express a muscle-like nicotinic receptor [8]. [125 I] α -Bgt binding to the nicotinic receptor in the TE671 cell line was measured on intact cells. Non-specific binding was checked in the presence of a high excess of unlabelled α -Bgt. No binding of [125 I] α -Bgt was detected on hepatoma PLC/PRF/5 (data not shown), a different human cell line. Gp120 from the HIV-1 strain IIIB was found to inhibit the binding of α -Bgt to TE671: 30% of maximum binding (B_0) was measured in the presence of 2×10^{-7} M gp120 (Fig. 2). Acetylcholine and nicotine were used under the same conditions to specifically inhibit the binding, as already described for α -Bgt binding to torpedo AChR [7,9]. HIV-1 gp120 inhibition of α -Bgt binding indicates a 'functional equivalence' between these proteins for nico-

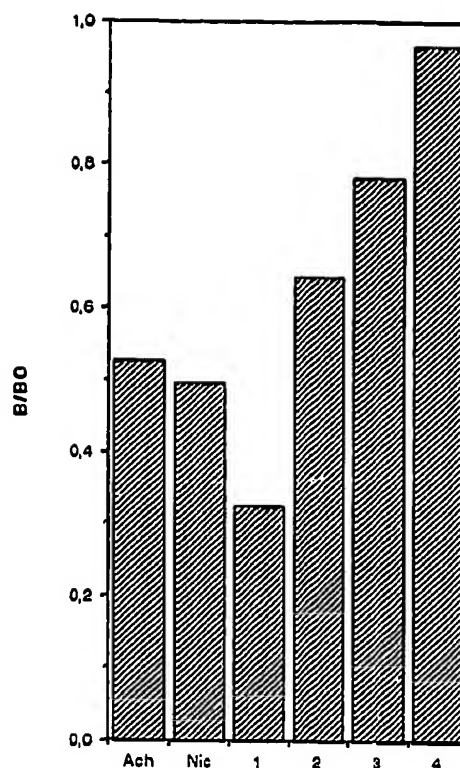


Fig. 2. [125 I] α -Bgt binding to TE671 AChR in the presence of (Ach) 2.5×10^{-2} M acetylcholine/ 2.5×10^{-4} M neostigmine; (Nic) 10^{-3} M nicotine; (1) 2×10^{-7} M gp120; (2) 10^{-7} M gp120; (3) 5×10^{-8} M gp120; (4) 2.5×10^{-8} M gp120. Each point is the mean of duplicate determinations after subtraction of non-specifically bound radioactivity (B), (B_0). Maximum binding (see text).

tinic receptor binding. To investigate whether the sequence of HIV-1 gp120, homologous to snake neurotoxins and rabies virus glycoprotein, might be involved in the binding of gp120 to AChR, we synthesized a 14-amino acid peptide (HG165-78: Asn-Ile-Ser-Thr-Ser-Ile-Arg-Gly-Lys-Val-Gln-Lys-Glu-Tyr), reproducing the sequence 165–178 of gp120. Once conjugated to KLH this peptide inhibits [125 I] α -Bgt binding to intact TE671 cells (Fig. 3). About 40% of maximum binding was obtained in the presence of 1.4×10^{-7} M conjugated peptide, but we could not measure any significant inhibition with the free peptide.

The effect of the protein carrier is not surprising. It has been reported in other cases and attributed to the stabilization of the peptide active conformation [10]. In our case the further ability of gp120 to inhibit the binding of α -Bgt to the nicotinic receptor in TE671 seems to indicate that the HG165-178 active conformation is similar to the one this sequence assumes in the native protein. Moreover immunization of mice with KLH-conjugated HG165-178 gave rise to an antiserum which bound to gp120 and also recognized rabies virus glycoprotein and α -Bgt in ELISA (Fig. 4), confirming

C-D-I-F-T-N-S-R-G-K-R	RV glycoprotein (residues 189-199)
F-N-I-S-T-S-I-R-G-K-V	HIV-1 gp120 (residues 164-174)
C-D-A-F-C-S-I-R-G-K-R	α -cobratoxin (residues 30-40)
C-D-A-F-C-S-S-R-G-K-V	α -bungarotoxin (residues 30-40)

Fig. 1. Sequence homology of HIV-1 gp120 with rabies virus glycoprotein and snake venom neurotoxins.

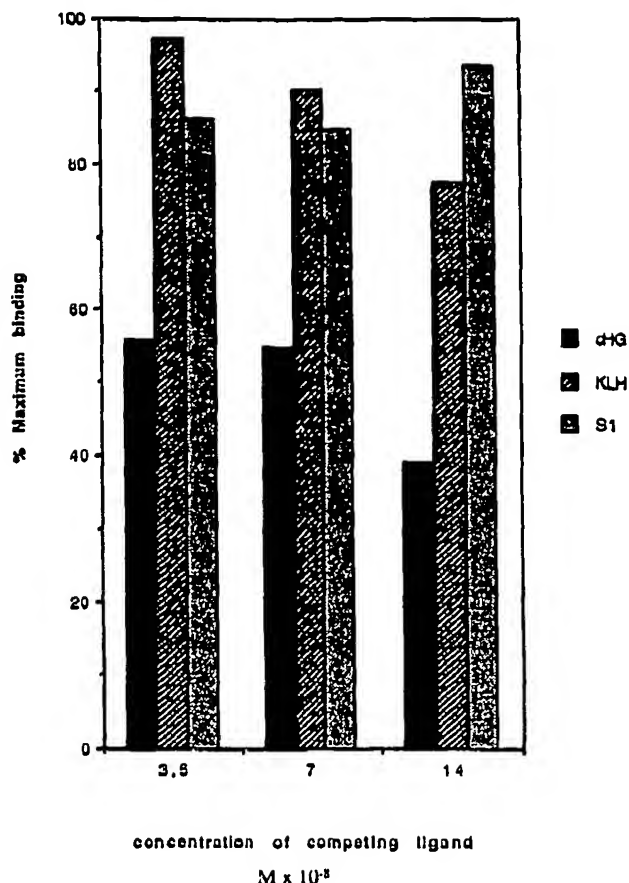


Fig. 3. [¹²⁵I]α-Bgt binding to TE671 AChR in the presence of different concentrations of the following competing ligands: KLH-conjugated HG165-178 (cHG), KLH, and an uncorrelated 14-amino acid KLH-conjugated peptide (S1).

the possibility of a structural similarity between the regions of these proteins having remarkable sequence homology. In the light of our results we cannot exclude the possibility that inhibition of α-Bgt binding to TE671 cells by HIV-1 gp120 is due, at least in part, to receptor down-regulation following gp120 binding.

The existence of an HIV-1 receptor alternative to CD4 in neurones and muscle cells is strongly suggested by evidence of the ability of HIV-1 to infect CD4-negative muscle and neural cells [11,12] and the lack of inhibition by soluble CD4 of HIV-1 infection of muscle and neuronal cell lines [13]. Galactosyl ceramide has been reported to specifically bind HIV-1 gp120, and has been proposed as an essential component of HIV-1 receptors in neural cell lines and brain cells expressing this or a related lipid [14,15]. Our evidence of the binding of gp120 to TE671 nicotinic receptors helps to explain HIV-1 infection of muscle cell lines, and suggests that nicotinic receptors may also bind HIV-1 gp120 in neural cells. At least two populations of nicotinic receptors are

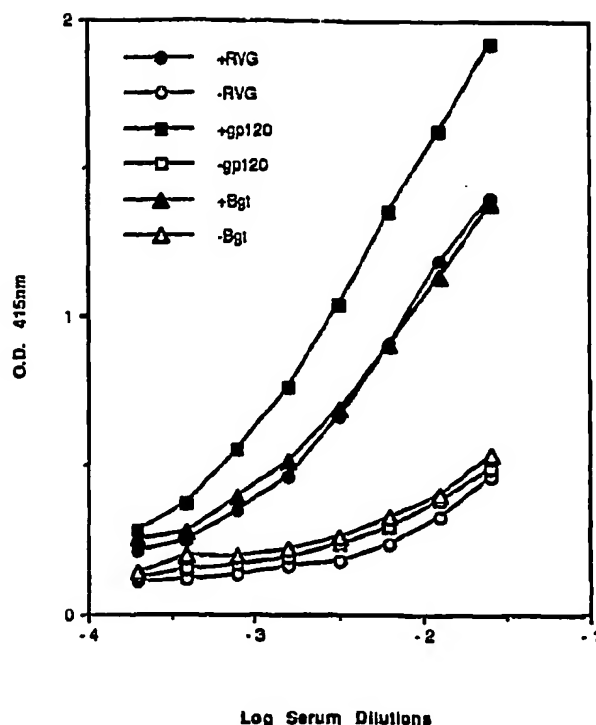


Fig. 4. Anti-KLH-conjugated HG165-178 mouse polyclonal antibodies tested in ELISA on RVG (+RVG), gp120 (+gp120) and α-Bgt (+Bgt), compared to equivalent dilution of normal mouse serum (-RVG), (-gp120) and (-Bgt).

expressed in the nervous system [16], one of which is labelled by α-Bgt. Moreover some of the cell lines, such as RD, TE671 and IMR32, in which a CD4-independent infection by HIV-1 has been proposed, are known to express α-Bgt binding nicotinic receptors [8,17].

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followed when assessing the new formulation. Following an overnight abstinence from other anticholinergic drugs, he administered 1 spray sublingually (equivalent to 0.1 ml volume). He noted improved pain and strength approximately 15 minutes post administration. Administration of either solution throughout the day provided satisfactory improvements in strength, endurance and relief from pain equivalent to prior therapeutic modalities. These observations confirm the importance of the nAChR binding properties of both formulations. The patient has employed oral and injectable formulations of the modified neurotoxin for over 3 years. Electromyograph recordings have indicated that the rate of deterioration associated with the disease has reduced significantly.

EXAMPLE 6

Human Subject with MS

[0071] A human volunteer with confirmed MS was administered oxidized alpha-cobratoxin in an oral formulation comprising 500 mcg/ml of the neurotoxin and 0.007% Benzalkonium chloride suspended in 0.9% physiological saline. In the absence of anticholinergic therapy the patient reported stiffness and pain upon rising and leg pain during the day. This combined with reduced endurance and strength comprised the symptoms to be followed when assessing the new formulation. Following an overnight abstinence from other anticholinergic drugs, he administered 1 spray sublingually (equivalent to 0.1 ml volume). He noted improved pain and strength approximately 15 minutes post administration. Administration of the solution throughout the day provided satisfactory improvements in strength, endurance and relief from pain equivalent to prior therapeutic modalities. Following 3 years of use, the patient continues to employ this product and reports his disease has stabilized and the rates of deterioration has significantly declined.

EXAMPLE 7

Human subject with MS

[0072] A human volunteer with confirmed MS was administered oxidized alpha-cobratoxin in a parenteral formulation comprising 500 mcg/ml of the neurotoxin and 0.001% Benzalkonium chloride suspended in 0.9% physiological saline. In the absence of anticholinergic therapy the patient reported stiffness and pain upon rising and leg pain during the day. This combined with reduced endurance and strength comprised the symptoms to be followed when assessing the new formulation. Following an overnight abstinence from other anticholinergic drugs, she administered 1 injection (equivalent to 0.5 ml volume). She noted improved pain and strength approximately 20 minutes post administration. Administration of the solution throughout the day provided satisfactory improvements in strength, endurance and relief from pain equivalent to prior therapeutic modalities. Following 3 years of use, the patient continues to employ this product and reports her disease has stabilized.

EXAMPLE 8

Human Subject with Adrenomyeloneuropathy (AMN)

[0073] A human volunteer with confirmed AMN was administered oxidized alpha-cobratoxin in an injectable for-

mulation comprising 600 mcg/ml of the neurotoxin and 0.01% Benzalkonium chloride suspended in 0.9% physiological saline. In the absence of anticholinergic therapy the patient reported reduced strength and poor endurance. This combined with reduced endurance and strength comprised the symptoms to be followed when assessing new formulations. Administration of the solution (0.2 cc t.i.d.) throughout the day provided satisfactory improvements in strength and endurance. Measured conduction velocities were recorded as improved over scores recorded prior to the initiation of therapy. This data strongly indicates the drug(s) are modulating the signals generated by the nerve cells and most reasonably through their interaction with nAChRs. The patient continues to employ this product and reports disease stabilization with treatment over 2 years.

[0074] While the invention has been described, and disclosed in various terms or certain embodiments or modifications which it has assumed in practice, the scope of the invention is not intended to be, nor should it be deemed to be, limited thereby and such other modifications or embodiments as may be suggested by the teachings herein are particularly reserved especially as they fall within the breadth and scope of the appended claims.

What is claimed is:

1. A method of treatment of animals suffering from neurological disorders comprising administering to the animal a disease mitigating dosage of a detoxified and neurotrophically active modified alpha-neurotoxin composition which targets nicotinic acetylcholine receptors.
2. The method of claim 1 wherein the detoxified and neurotrophically active modified composition comprises a fraction containing the alpha-neurotoxins.
3. The method of claim 1 wherein the alpha-neurotoxins are selected from the group consisting of alpha-bungarotoxin, kappa-bungarotoxin, alpha-cobratoxin, alpha-cobrotoxin, alpha-conotoxins (G1, M1, S1, S1A, Im1), alpha-dendrotoxin and crebutoxin.
4. The method of claim 1 wherein the alpha-neurotoxin composition comprises alpha-cobratoxin.
5. The method of claim 1 wherein in humans the dosage of the composition is from about 0.05 to 10 ml based on a 0.1% solution of the modified cobratoxin per 150 lbs body weight.
6. The method of claim 5 wherein the dosage is from 0.4 to 3 ml.
7. The method of claim 5 wherein the dosage is administered in a frequency of from every other week to daily.
8. The method of claim 5 wherein the dosage is administered at least weekly.
9. The method of claim 5 wherein the dosage is administered at least daily.
10. The method of claim 5 wherein composition administration methods include by injection (subcutaneous, intramuscular and intravenous), orally, otically and by intradermal routes.
11. The method of claim 1 wherein subject neurological condition benefits from improved nerve conduction and modulation.
12. The method of claim 11 wherein the neurological condition is selected from the group comprising Amyotrophic Lateral Sclerosis, other spinal atrophies, Multiple Sclerosis, Myasthenia Gravis, Muscular Dystrophy, Leukodystrophies, Adrenomyeloneuropathy and Ataxias.

13. A method of vaccinating a subject comprising administering to the subject an immunogenic amount of a detoxified and neurotropically active modified neurotoxin composition with or without the inclusion of an adjuvant.

14. The method of claim 13 wherein composition administration methods include by injection (subcutaneous, intramuscular and intravenous), orally, otically and by intradermal routes.

15. The method of claim 13 wherein blocking neurotropic viruses that employ the nAChR for cell entry comprises administering to a subject an amount of a detoxified and neurotropically active modified neurotoxin composition.

16. The method of claim 15 wherein composition administration methods include by injection (subcutaneous, intra-

muscular and intravenous), orally, otically and by intradermal routes.

17. A composition comprising an administrable form of a detoxified and neurotropically active modified snake venom neurotoxin wherein a Naja venom neurotoxin is alpha-cobratoxin and the composition is atoxic.

18. The composition of claim 17, wherein the alpha-cobratoxin can be administered orally when combined in a solution with benzalkonium chloride.

19. The composition according to claim 18, wherein the alpha-cobratoxin can be administered orally when combined in a solution with benzalkonium chloride at a protein:detergent ratio of between 1:6 to 1:8, and preferably 1:7.5.

* * * * *

What is claimed is:

1. A composition for preventing HIV infection of mammalian cells, the composition comprising an anti-immunodeficiency virus immunokine capable of binding to a cellular protein in a manner that prevents HIV infection of the cell.
2. A composition according to claim 1 wherein the immunodeficiency virus is selected from the group consisting of HIV-1, HIV-2 and SIV.
3. A composition according to claim 1 wherein the immunokine comprises an inactivated bioactive polypeptide.
4. A composition according to claim 3 wherein the inactivated bioactive polypeptide comprises a toxin selected from neurotoxins affecting the presynaptic neurojunction, toxins affecting postsynaptic neurojunction, and toxins affecting ion channels.
5. A composition according to claim 4 wherein the toxin comprises α -cobratoxin.
6. A composition according to claim 1 wherein the immunokine is adapted to bind one or more of a chemokine receptor protein, and a cellular cofactor for a cellular HIV receptor protein.
7. A composition according to claim 6 wherein the protein to which the immunokine of the invention binds is selected from the group consisting of CD4, CXCR4 and CCR5, consisting of CD4 and CXCR4 or CCR5.
8. A composition according to claim 3 wherein the immunokine provides a substantially native toxin structure wherein one or more of the disulfide bridges are lacking by a method selected from the ozonation of native toxin, genetic engineering, and protein synthesis.
9. A composition according to claim 8 wherein ozonation is performed in a stoichiometric manner.
10. A composition according to claim 9 wherein the immunokine comprises inactivated alpha-cobratoxin in which the disulfide bridges are substantially lacking by ozonation of native alpha-cobratoxin.

11. A method of inhibiting infection of a cell by HIV comprising adding to the cell an anti-immunodeficiency virus immunokine capable of binding to a cellular protein on the cell, wherein upon binding of the immunokine to the cellular protein infection of the cell by HIV is inhibited.

12. A method of treating HIV infection in a human comprising administering to the human an anti-immunodeficiency virus immunokine capable of binding to a cellular protein on a cell, wherein upon binding of the immunokine to the cellular protein, infection of the cell by HIV is inhibited.

13. A method of preparing an anti-immunodeficiency virus immunokine capable of binding to a cellular protein on a cell, the method comprising the chemical, genetic and synthetic modification of native neurotoxins.

14. A method of identifying a target cell for immunodeficiency virus infection, the method comprising adding to a population of cells an anti-immunodeficiency virus immunokine capable of binding to a cellular protein on a cell, wherein binding of the immunokine to a cell in the population is an indication that the cell is an immunodeficiency virus target cell.

15. A method of identifying a candidate anti-immunodeficiency virus compound, the method comprising isolating a test compound capable of binding to an anti-immunodeficiency virus immunokine, which immunokine binds to a cellular protein, and assessing the ability of the test compound to inhibit infection of a cell by an immunodeficiency virus in an antiviral assay, wherein inhibition of infection of the cell by the immunodeficiency virus in the presence of the test compound is an indication that the test compound is an anti-immunodeficiency virus compound.

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DOCUMENT-IDENTIFIER: US 20030211465 A1

TITLE: Immunokine composition and method

Abstract Paragraph - ABTX (1):

A composition and method for preventing HIV infection of mammalian cells. One aspect of the invention relates to an anti-immunodeficiency virus immunokine capable of binding to a cellular protein in a manner that prevents HIV infection of that cell. The compositions can include either an active bioactive polypeptide, such as native cobratoxin, and/or an inactivated bioactive polypeptide, such as cobratoxin in which one or more of the native disulfide bridges have been prevented from forming. The term "immunokine" is used to refer to an inactivated bioactive polypeptide, whether inactivated by chemical, genetic, and/or synthetic means as described herein, with the proviso that a corresponding active bioactive polypeptides can be included where applicable (e.g., for in vitro use).

Pre-Grant Publication (PGPub) Document Number - PGNR (1):

20030211465

Title - TTL (1):

Immunokine composition and method

Summary of Invention Paragraph - BSTX (21):

[0019] The present invention provides a composition and method for preventing HIV infection of mammalian cells. One aspect of the invention relates to an anti-immunodeficiency virus immunokine capable of binding to a cellular protein in a manner that prevents HIV infection of that cell. In another aspect, the immunodeficiency virus is selected from the group consisting of HIV-1, HIV-2 and SIV. In another aspect, the invention relates to the identification of a biologic anticholinergic agent capable of binding to a cellular protein in a manner that prevents HIV infection of that cell. In yet another aspect the invention relates to an anti-immunodeficiency virus immunokine derived from a biologic anticholinergic agent which can be administered in vivo for the treatment of HIV infection. The immunodeficiency virus can be selected from the group consisting of Lentiviruses (HIV-1, HIV-2, SIV, EIAV, BIV, FIV and FeLV).

Summary of Invention Paragraph - BSTX (22):

[0020] Compositions of this invention can include either an "active bioactive polypeptide", such as native cobratoxin, and/or an "inactivated bioactive polypeptide", such as cobratoxin in which one or more of the native disulfide bridges have been prevented from forming. While not presently preferred for in vivo applications, it appears that the active polypeptides exhibit the desired antiviral activity, and in turn, can be used for in vitro (e.g., diagnostic) applications. The term "immunokine" will generally be used to refer to an inactivated bioactive polypeptide, whether inactivated by chemical, genetic, and/or synthetic means as described herein, with the proviso that a corresponding active bioactive polypeptides can be included where applicable (e.g., for in vitro use).

Summary of Invention Paragraph - BSTX (24):

[0022] Proteins such as those from venoms, as described herein, have long been recognized for their ability to bind to specific receptors on the surface of human cells. These neurospecific proteins bind to such common receptors as the acetylcholine receptor for example. Significantly less well known than the interactions between venom proteins and human cells is the ability of these venoms to cause cells to migrate toward or in response to the venom proteins. This cellular activity is called chemotaxis and, until the characterization of these venom proteins by the present Applicants, this property has only been attributed to compounds called chemokines produced in immune cells. For these reasons, we will heretofore refer to our venom proteins as "immunokines".

Summary of Invention Paragraph - BSTX (25):

[0023] In yet another aspect of the invention, the protein to which the immunokine of the invention binds is one or more of a chemokine receptor protein, preferably, an HIV receptor protein and/or a cellular cofactor for a cellular HIV receptor protein. More preferably, the protein to which the immunokine of the invention binds is selected from the group consisting of CD4, CXCR4 and CCR5; and most preferably, the protein to which the immunokine binds is CD4/CXCR4 and/or CD4/CCR % complexes.

Summary of Invention Paragraph - BSTX (26):

[0024] In another aspect of the invention, the immunokine is most preferably selected from the group consisting of post-synaptic alpha-neurotoxins (Group II) and anticholinergic peptides.

Summary of Invention Paragraph - BSTX (27):

[0025] The invention also relates to an isolated DNA encoding an immunokine capable of binding to a cellular protein in the manner described herein.

Summary of Invention Paragraph - BSTX (28):

[0026] The invention also relates to a method of inhibiting infection of a cell by HIV comprising adding to the cell an anti-immunodeficiency virus immunokine capable of binding to a cellular protein on the cell, wherein upon binding of the immunokine to the cellular protein infection of the cell by HIV is inhibited.

Summary of Invention Paragraph - BSTX (29):

[0027] Also included in the invention is a method of treating HIV infection in a human comprising administering to the human an anti-immunodeficiency virus immunokine capable of binding to a cellular protein on a cell, wherein upon binding of the immunokine to the cellular protein, infection of the cell by HIV is inhibited, thereby treating the HIV infection in the human.

Summary of Invention Paragraph - BSTX (30):

[0028] The invention further includes a method of obtaining an anti-immunodeficiency virus immunokine capable of binding to a cellular protein on a cell, in one embodiment the method comprising an oxidative process for the chemical production of immunokine by combining ozone with the protein of interest, e.g., a native or synthetic neurotoxin.

Summary of Invention Paragraph - BSTX (31):

[0029] Also included in the invention is a method of identifying a target cell for immunodeficiency virus infection, the method comprising adding to a population of cells native or synthetic active bioactive polypeptide (e.g., alpha-cobratoxin) or an anti-immunodeficiency virus immunokine capable of binding to a cellular protein on a cell, wherein binding of the immunokine to a cell in the population is an indication that the cell is an immunodeficiency virus target cell.

Summary of Invention Paragraph - BSTX (32):

[0030] In addition, there is provided a method of identifying a candidate anti-immunodeficiency virus compound. This method comprises isolating a test compound capable of binding to an active bioactive polypeptide such as alpha-cobratoxin or an anti-immunodeficiency virus immunokine, which immunokine binds to a cellular protein, and assessing the ability of the test compound to inhibit infection of a cell by an immunodeficiency virus in an antiviral assay, wherein inhibition of infection of the cell by the immunodeficiency virus in the presence of the test compound is an indication that the test compound is an anti-immunodeficiency virus compound.

Summary of Invention Paragraph - BSTX (34):

[0031] In one preferred embodiment, the invention relates to an antiviral, anticholinergic protein and immunokine which binds to one or more cellular proteins essential for entry of a virus into a cell expressing that protein. The immunokine of the invention is an antiviral immunokine in that it is an immunokine which binds to one or more cellular proteins that are essential for virus entry into the cell in which the cellular protein is expressed. By binding to the cellular protein, the immunokine of the invention inhibits entry of the virus into the cell and is therefore termed an antiviral immunokine despite the fact that it does not bind to a viral protein, but rather, binds to a cellular protein. The invention further relates to an antiviral immunokine which binds to one or more cellular proteins essential for entry of a virus into a cell expressing that protein.

Summary of Invention Paragraph - BSTX (35):

[0032] The virus against which the antiviral immunokine is directed is an immunodeficiency virus, that is, a virus which causes an immunodeficiency disease. Thus, the immunokine of the invention is termed an anti-immunodeficiency virus immunokine. Such immunodeficiency virus should be construed to include any strain of HIV or SIV, as well as other lentiviruses (FIV, FeLV, BIV, and EIAV).

Summary of Invention Paragraph - BSTX (37):

[0034] Without intending to be bound by theory, it appears that both native alpha-cobratoxin and an immunokine of the invention are each capable of binding to a cellular protein required to form a functional cellular receptor for entry of HIV into a cell. In one preferred embodiment, the immunokine of the invention is an immunokine which binds to a cellular receptor and/or to a cellular co-factor required for entry of HIV into a cell. A "cellular co-factor" as used herein, is defined as a protein which is required, in association with a cellular receptor for HIV, for entry of HIV into cells.

Summary of Invention Paragraph - BSTX (38):

[0035] According to the invention, the polypeptides (e.g., native or immunokine) of the invention is useful in a method of inhibiting infection of a cell by HIV as described herein. Moreover, the immunokine of the invention is useful in a method of screening compounds for anti-HIV activity as described herein. Additional uses for alpha-cobratoxin or an immunokine of the invention include the identification of cells in the body which are potential targets for viral

infection. The immunokine is thus also useful for the isolation of such cells using flow cytometry technology or other cellular isolation techniques which are common in the art. The invention also relates to methods of use of the immunokine of the invention, which methods include diagnostic and therapeutic uses.

Summary of Invention Paragraph - BSTX (39):

[0036] By "antiviral activity" as used herein, is meant an immunokine which when added to an immunodeficiency virus or to a cell to be infected with such a virus, mediates a reduction in the ability of the virus to infect and/or replicate in the cell compared with the ability of virus to infect and/or replicate in the cell in the absence of the immunokine. Examples of assays for antiviral activity are described in detail in the experimental detail section and include, but are not limited to, reverse transcriptase assays, immunofluorescence assays, assays for formation of syncytia, antigen capture assays and the like.

Summary of Invention Paragraph - BSTX (40):

[0037] Immunokine Preparation

Summary of Invention Paragraph - BSTX (41):

[0038] A composition of this invention can be prepared in any suitable manner. For instance, native cobratoxin can be obtained and used in its native (e.g., unmodified) form, and is shown to inhibit HIV infection of cells (PMNC) with a similar efficacy to the corresponding immunokine described herein. Toxins themselves can be chemically modified (e.g., using ozone, performic acid, iodoacetamide etc.), and other cobratoxin homologues (see Group II) can be prepared. Toxin modifications include site-directed mutants (mono and poly-substituted mutants such as tryptophan, tyrosine, lysine and arginine), chimeras and other homologous peptide fragments produced from the parent protein through genetic engineering or synthetic peptide production.

Summary of Invention Paragraph - BSTX (42):

[0039] An inactivated bioactive polypeptide (e.g., immunokine) of this invention can be prepared using any suitable means. As described herein, the immunokine can be chemically produced in an oxidative process in combination with the protein of interest, e.g., a neurotoxin. The use of ozone treatment to prepare the immunokine is particularly preferred, e.g., in view of the simplicity of manufacture, the modest facility requirements and self sterilizing nature of the production procedure. Under controlled conditions, ozone specifically modifies certain amino-acids such as methionine, cysteine and tryptophan to methionine sulphone, cysteic acid and kynurenine respectively. Cobratoxin has no methionine, ten (10) cysteine and one (1) tryptophan residues.

Summary of Invention Paragraph - BSTX (43):

[0040] Other procedures can be used as well, though these with each such procedure providing a product that varies in its relative potencies when compared to immunokine produced with ozone. Those procedures include the use of hydrogen peroxide, performic acid, carboxyamidomethylation, iodoacetamide, iodoacetic acid and Oxone (Caro's Acid) but includes any chemical agent that acts as an oxidizer or alkylator that can render proteins like cobratoxin atoxic and suitable for administration to a host. The circumstances where a difference procedure would be employed would be if the resultant product demonstrated better therapeutic activity in other applications, for example superior immuno-modulatory, anti-tumor or anti-viral activity, but they emphasize the importance of breaking the disulphide bonds with a concomitant conformational reorganization similar to that during disulphide oxidation. The requirement for

scission of all the disulphide bonds for optimal function has not yet been fully investigated but sufficient bonds must be broken to render a protein like alpha-cobratoxin safe for administration to a host.

Summary of Invention Paragraph - BSTX (49):

[0046] In one aspect, the present invention provides a method of preparing a parenteral composition comprising an immunokine (e.g., an immunokine), the method comprising the steps of:

Summary of Invention Paragraph - BSTX (54):

[0051] In another aspect, the invention provides a composition comprising an immunokine that has been rendered inactive by virtue of the failure to form one or more of its disulfide bridges. In a related aspect, the invention provides a composition for in vivo administration comprising a bioactive immunokine that has been inactivated in the manner described herein.

Summary of Invention Paragraph - BSTX (55):

[0052] The method can be used to prepare immunokines from, or based upon, a variety of natural compounds, including "Group I neurotoxins" (namely, toxins affecting the presynaptic neurojunction), Group II neurotoxins (namely those affecting the postsynaptic neurojunction), and Group III neurotoxins (those affecting ion channels). cDNA sequences for such polypeptides are generally known, or can be determined using conventional techniques.

Summary of Invention Paragraph - BSTX (58):

[0055] Preferably, the cDNA is expressed using a microbial expression system, such as Escherichia coli, Saccharomyces cerevisiae and Pichia pastoris. From a safety and environmental perspective it is preferable that the cDNA is expressed in a microbial expression system under conditions in which the product is cytoplasmically produced, as opposed to extracellularly secreted. In an exemplary embodiment, the immunokine is expressed using a microbial expression system, under conditions in which the leader sequence of naturally-occurring cDNA is removed and replaced with only the initiation codon.

Summary of Invention Paragraph - BSTX (59):

[0056] Immunokines of the present invention are generally stable under suitable conditions of storage and use in which the disulfide bonds are prevented from spontaneously reforming, or are allowed to reform in a manner that precludes the undesirable activity of the immunokine. Optionally, and preferably, once the inactive polypeptide has been recovered, it is treated by suitable means to ensure that the cysteine residues do not spontaneously reform to form disulfide bridges. An example of a preferred treatment means is the use of ozone treatment as described herein.

Summary of Invention Paragraph - BSTX (60):

[0057] In another optional, and alternative, embodiment a immunokine such as neurotoxin is produced in an inactive form using the Pichia expression system described herein. To the best of Applicants knowledge, the prior art fails to teach or suggest the preparation of a toxin in inactive form by the route of cytoplasmic expression in yeast.

Summary of Invention Paragraph - BSTX (61):

[0058] The method and composition of the present invention provide a unique and valuable tool for the synthesis and recovery of bioactive immunokines in a manner capable of diminishing undesirable activity, yet retaining other useful properties of the immunokine (such as immunogenicity and antiviral activity).

Summary of Invention Paragraph - BSTX (67):

[0064] The method of the present invention involves an initial step of identifying a bioactive immunokine having a tertiary structure in which bioactivity is dependent, at least in part, on the formation of one or more disulfide bridges between cysteine residues. Typically, the immunokine will be one that is naturally secreted in the course of its synthesis, since it is the secretion process that will provide the necessary posttranslational steps, including disulfide bond formation. Preferably, the immunokine is one that is stable when recovered and that retains other desirable properties in the unfolded state, such as immunogenicity and/or antiviral, anti-tumor or wound healing activity.

Summary of Invention Paragraph - BSTX (68):

[0065] The amino acid sequence and tertiary structure of a number of bioactive polypeptides is known. Suitable immunokines include those in which one or more disulfide bridges are known to form in the natural configuration, and in which such bridge(s) are necessary for the bioactivity of the immunokine. Such bridges can be of either an intramolecular (i.e., within a single polypeptide) nature and/or an intermolecular (e.g., between discrete subunits) nature.

Summary of Invention Paragraph - BSTX (72):

[0069] Immunokine components from animal venoms, for instance, can be obtained from the animals themselves or from other sources, or they can be created in the laboratory using conventional protein engineering techniques. In the former approach, animals are induced by mechanical or electrical stimuli to release venom from their glands, which travels through a venom canal and out the fang or stinger. The venom is collected and various constituents of the venom are purified by conventional chromatographic techniques.

Summary of Invention Paragraph - BSTX (73):

[0070] In the latter approach, constituents from the venom are synthesized by cloning the genes encoding the various immunokine elements and expressing these genes in heterologous host systems such as bacteria, yeast or higher eucaryotic cell lines. Yeast expression systems are presently preferred, since they tend to provide an optimal combination of such properties as yield and adaptability to human use products.

Summary of Invention Paragraph - BSTX (78):

[0075] Optionally, and preferably, the method provides a further step of treating the immunokines in order to retain the cysteine residues and prevent the spontaneous formation of disulfide bonds. A preferred treatment includes ozone treatment, in the manner described herein. Ozonation affects the cysteine residues by converting the pendent sulfhydryl (--SH) groups to corresponding --SO₃X groups, which, unlike the sulfhydryl groups, are unable to form a disulfide bridge. Such treatment is not necessary, however, for those inactivate polypeptides that are found to not spontaneously reform, and that provide the desired activity. Ozonation is preferred for polypeptides such as neurotoxins, where Applicant has shown that upon cleavage and ozonation of the sulfhydryl groups, native neurotoxins are both stable and active.

Summary of Invention Paragraph - BSTX (81):

[0078] Polypeptides such as the preferred neurotoxins and immunokines can be prepared using genetic engineering techniques within the skill of those in the art, given the present description. See, for instance, (Fiordalisi et al., (1996) Toxicon 34, 2, 213-224, Krajewski et al (1999) "Recombinant m1-toxin" presented at the 29.sup.th Annual Meeting of the Society for Neuroscience) and (Smith et al., (1997) Biochemistry, 36, no. 25, 7690-7996. As the native cobratoxin gene is available, a number of bioengineered variants can be prepared which replace the residues required for disulphide bond formation with other residues. As these amino acid substitutions must be expressed in vivo, the availability of modifications are typically limited to the use of native residues (the standard 20 naturally occurring amino acids) and the host to be employed for expression. In the host, the codon usage will be important in ensuring efficient and maximal expression of the novel protein. Theoretically any amino acid can be substituted for cysteine but as this is a more costly approach to generating immunokine variants relative to synthetic peptide techniques certain residues have been selected which best reproduce the protein characteristics resulting from chemical exposure.

Summary of Invention Paragraph - BSTX (83):

[0080] Cleavage of the native cobratoxin and immunokine protein can be achieved with serine proteases (i.e. trypsin) but at sites containing positive residues. This permits also the evaluation and production of smaller peptide fragments for biological activity. The conversion of cysteine to cysteic acid also permits the substitution by other acidic residues such as E, D, Q, N and S. The substitution of E and D for cysteine is estimated to produce a protein with a pI similar to that of alpha-immunokine (pI=4.5). The substitution of cysteine with the residues glycine and alanine would represent standard "neutral" substitutions. A suitable method for creating these genes has been described previously (Smith et al., (1997)). The codon usage of the DNA fragments is optimized for use in commercially used bacterial and yeast expression systems Escherichia coli and Pichia pastoris respectively.

Summary of Invention Paragraph - BSTX (84):

[0081] Given the advances in technology in cloning DNA encoding proteins comprising antibodies, the invention also includes DNA which encodes the immunokine of the invention, or a portion of such immunokine. The nucleic acid encoding the immunokine may be cloned and sequenced using technology which is available in the art, and is described, for example, in Wright et al. (1992, Critical Rev. in Immunol. 12(3,4):125-168) and the references cited therein. Further, the immunokine of the invention may be "humanized" using the technology described in Wright et al., (supra) and in the references cited therein.

Summary of Invention Paragraph - BSTX (85):

[0082] For example, to generate a phage immunokine library, a cDNA library is first obtained from mRNA which is isolated from cells, e.g., the hybridoma, which express the desired protein to be expressed on the phage surface, e.g., the desired immunokine. cDNA copies of the mRNA are produced using reverse transcriptase. cDNA which specifies immunoglobulin fragments are obtained by PCR and the resulting DNA is cloned into a suitable bacteriophage vector to generate a bacteriophage DNA library comprising DNA specifying immunoglobulin genes. The procedures for making a bacteriophage library comprising heterologous DNA are well known in the art and are described, for example, in Sambrook et al. (1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, N.Y.).

Summary of Invention Paragraph - BSTX (86):

[0083] Bacteriophage which encode the desired immunokine, e.g., an immunokine, may be engineered such that the protein is displayed on the surface thereof in such a manner that it is

available for binding to its corresponding binding protein, e.g., the antigen against which the immunokine is directed. Thus, when bacteriophage which express a specific immunokine are incubated in the presence of a cell which expresses the corresponding antigen, the bacteriophage will bind to the cell. Bacteriophage which do not express the immunokine will not bind to the cell. Such panning techniques are well known in the art and are described for example, in Wright et al., (supra).

Summary of Invention Paragraph - BSTX (87):

[0084] By the term "synthetic immunokine" as used herein, is meant an immunokine which is generated using recombinant DNA technology, such as, for example, an immunokine expressed by a bacteriophage as described herein. The term should also be construed to mean an immunokine which has been generated by the synthesis of a DNA molecule encoding the immunokine and which DNA molecule expresses an immunokine protein, or an amino acid sequence specifying the immunokine, wherein the DNA or amino acid sequence has been obtained using synthetic DNA or amino acid sequence technology which is available and well known in the art.

Summary of Invention Paragraph - BSTX (88):

[0085] The invention thus includes a DNA encoding the immunokine of the invention or a portion of the immunokine of the invention. To isolate DNA encoding an immunokine, for example, DNA is extracted from immunokine expressing phage obtained according to the methods of the invention. Such extraction techniques are well known in the art and are described, for example, in Sambrook et al. (supra).

Summary of Invention Paragraph - BSTX (92):

[0089] To obtain a substantially pure preparation of a protein comprising, for example, an immunokine, generated using the methods of the invention, the protein may be extracted from the surface of the phage on which it is expressed. The procedures for such extraction are well known to those in the art of protein purification. Alternatively, a substantially pure preparation of a protein comprising, for example, an immunokine, may be obtained by cloning an isolated DNA encoding the immunokine into an expression vector and expressing the protein therefrom. Protein so expressed may be obtained using ordinary protein purification procedures well known in the art.

Summary of Invention Paragraph - BSTX (94):

[0091] Current techniques in peptide chemistry allow for proteins in excess of 80 amino acids can be reliably produced using automated Fmoc solid phase synthesis (ABI 433A Peptide Synthesizer, Perkin Elmer--see www.perkin-elmer.com). Non-native amino acids (acetamidomethyl cysteine, carboxyamidomethyl cysteine, cysteic acid, kynurenine and methionine sulphone) are acquired from Advanced Chemtech (Louisville, Ky.) or Quchem (Belfast, Ireland). Other oxidized or alkylated amino acid variants are available from these agents. The generation of alpha-immunokine is achieved by substituting primarily the cysteine residues (from 1 pair to all 5 disulphide couples) with those residues described above to mimic the effects of ozone and other chemical modifications. Furthermore the substitution of other native and non-native residues for cysteine can be investigated in an attempt to identify immunokine variants with improved biological activity. Also peptide fragments from within the cobratoxin sequence can be created (analogous to Hinmann et al., (1999), Immunoparmacol. Immunotoxicol, 21 (3), 483-506) and examined for receptor binding activity.

Summary of Invention Paragraph - BSTX (95):

[0092] Inactivated bioactive polypeptides of this invention can be formulated and delivered in any suitable manner. For instance, for use in treating existing HIV infections, an immunokine will typically be provided in a substantially pure and sterile form, and in a vehicle adapted for delivery. As used herein, the term "substantially pure" describes a compound, e.g., a protein or polypeptide which has been separated from components which naturally accompany it. Typically, a compound is substantially pure when at least 10%, more preferably at least 20%, more preferably at least 50%, more preferably at least 60%, more preferably at least 75%, more preferably at least 90%, and most preferably at least 99% of the total material (by volume, by wet or dry weight, or by mole percent or mole fraction) in a sample is the compound of interest. Purity can be measured by any appropriate method, e.g., in the case of polypeptides by column chromatography, gel electrophoresis or HPLC analysis. A compound, e.g., a protein, is also substantially purified when it is essentially free of naturally associated components or when it is separated from the native contaminants which accompany it in its natural state.

Summary of Invention Paragraph - BSTX (96):

[0093] To inhibit infection of cells by HIV in vitro, cells are treated with the immunokine of the invention, or a derivative thereof, either prior to or concurrently with the addition of virus. Inhibition of infection of the cells by the immunokine of the invention is assessed by measuring the replication of virus in the cells, by identifying the presence of viral nucleic acids and/or proteins in the cells, for example, by performing PCR, Southern, Northern or Western blotting analyses, reverse transcriptase (RT) assays, or by immunofluorescence or other viral protein detection procedures. The amount of immunokine and virus to be added to the cells will be apparent to one skilled in the art from the teaching provided herein.

Summary of Invention Paragraph - BSTX (97):

[0094] To inhibit infection of cells by HIV in vivo, the immunokine of the invention, or a derivative thereof, is administered to a human subject who is either at risk of acquiring HIV infection, or who is already infected with HIV. Prior to administration, the immunokine, or a derivative thereof, is suspended in a pharmaceutically acceptable formulation such as a saline solution or other physiologically acceptable solution which is suitable for the chosen route of administration and which will be readily apparent to those skilled in the art of immunokine preparation and administration. The dose of immunokine to be used may vary dependent upon any number of factors including the age of the individual, the route of administration and the extent of HIV infection in the individual. The immunokine is prepared for administration by being suspended or dissolved in a pharmaceutically acceptable carrier such as saline, salts solution or other formulations apparent to those skilled in such administration.

Summary of Invention Paragraph - BSTX (98):

[0095] Typically, the immunokine is administered in a range of 0.1 microgram to 1 g of protein per dose. Approximately 1-10 doses are administered to the individual at intervals ranging from once per day to once every few years. The immunokine may be administered by any number of routes including, but not limited to, subcutaneous, intramuscular, oral, intravenous, intradermal, intranasal or intravaginal routes of administration. The immunokine of the invention may be administered to the patient in a sustained release formulation using a biodegradable biocompatible polymer, or by on-site delivery using micelles, gels and liposomes, or rectally (e.g., by suppository or enema). The appropriate pharmaceutically acceptable carrier will be evident to those skilled in the art and will depend in large part upon the route of administration.

Summary of Invention Paragraph - BSTX (99):

[0096] The immunokine (including the corresponding active bioactive polypeptide) of the invention may also be used in a method of screening compounds for anti-HIV activity. A test compound is first screened for the ability to bind to the immunokine of the invention. Compounds which bind to the immunokine are likely to share structural and perhaps biological activities with CXCR4 and thus, may serve as competitive inhibitors for inhibition of the interaction of HIV envelope protein with CD4 and/or CXCR4 plus CD4. An immunokine-binding compound is further tested for antiviral activity by treating cells with the compound either prior to or concurrently with the addition of virus to the cells. Alternatively, the virus and the compound may be mixed together prior to the addition of the mixture to the cells. The ability of the compound to affect virus infection is assessed by measuring virus replication in the cells using any one of the known techniques, such as a RT assay, immunofluorescence assays and other assays known in the art useful for detection of viral proteins or nucleic acids in cells. Generation of newly replicated virus may also be measured using known virus assays such as those which are described herein.

Summary of Invention Paragraph - BSTX (100):

[0097] The immunokine of the invention may also be used in competition assays to screen for compounds that bind to CXCR4 and which therefore prevent binding of the immunokine to CXCR4. Such compounds, once identified, may be examined further to determine whether or not they prevent entry of virus into cells. Compounds which prevent entry of virus into cells are useful as anti-viral compounds.

Summary of Invention Paragraph - BSTX (101):

[0098] Additional uses for the immunokine of the invention include the identification of cells in the body which are potential targets for infection by an immunodeficiency virus.

Summary of Invention Paragraph - BSTX (103):

[0100] Cells which are potential targets for HIV infection may be identified by virtue of the presence of CXCR4 on their surface. The immunokine of the invention facilitates identification of these cells as follows: The immunokine of the invention is first combined with an identifiable marker, such as an immunofluorescent or radioactive marker. Cells which are obtained from a human subject are then reacted with the tagged immunokine. Binding of the immunokine to cells is an indication that such cells are potential targets for HIV infection. The identification of cells which may be infected with HIV is important for the design of therapies for the prevention of HIV infection. For example, CXCR4 is differentially expressed and regulated on human T lymphocytes (Bleul et al., 1997, Proc. Natl. Acad. Sci. USA 94:1925-1930). Further, reactivity of immune cells to MAb 12G5 is high on naive cells and low on memory cells and thus, the pattern of expression of CXCR4 and its utilization by viruses may contribute to immune dysfunction. CXCR4 has also been detected, using the immunokine of the invention, on some endothelial cells (in atherosclerotic plaques), platelets and some hematopoietic precursor cells. In the case of individuals who are infected with HIV, the identification of target cells provides an immune profile of these individuals which provides useful information regarding the progress of their infection.

Summary of Invention Paragraph - BSTX (104):

[0101] In addition to the aforementioned uses for the immunokine of the invention, the immunokine is useful for the detection of CXCR4 on a variety of cell types on which CXCR4 may be expressed. For example, CXCR4 is expressed on human neurons (Hesselgesser et al., 1997, Current Biology 7:112-121), including cells in the human brain.

Detail Descriptive Paragraph - DETX (84):

Immunokine Production

Detail Description Paragraph - DETX (85):

[0168] A preferred process for the production of an immunokine of this invention is outlined below. Alpha-immunokine-NNS (immunokine) is a protein derived from alpha-cobratoxin. Cobratoxin (CTX) has a molecular weight of 7821 and is composed of 71 amino acids. The native protein is purified from the venom of the Thailand cobra, *Naja naja siamensis*. Alpha-cobratoxin from the Thailand cobra (*Naja naja siamensis*) was purchased from Biotoxins, Kississimi, Fla. The published amino-acid sequence for cobratoxin employing single letter code is:

Detail Description Paragraph - DETX (87):

[0170] The procedure below describes the dissolution of ozone into saline (0.9%) and its addition to cobratoxin to form immunokine. The reaction is rapid being completed in minutes. In order to create a more homogeneous product consistently the procedure described below was developed whereby an ozone-saturated fluid is added directly to a solution of cobratoxin. It is expected that greater reproducibility can be achieved with this method. The critical point of the reaction centers on adding sufficient ozone to ensure that no native cobratoxin remains. When the reaction is deemed complete several parameters can be measured to be suggestive of successful preparation. The reaction can be conducted at ambient temperatures but the concentration of the final product is limited to below 350 mg/ml. This arises because of the limitations placed on dissolving ozone in saline at these temperatures.

Detail Description Paragraph - DETX (119):

[0201] An immunokine solution prepared in this manner had an acidic pH and a pI of approximately 4.5. Cobratoxin solutions are basic having pH of 8.5. In solution, the drug migrates through molecular sieving gels as monomers, dimers and tetramers. Cobratoxin migrates under these conditions as a monomer. Upon analysis on NuPAGE (Novex) SDS polyacrylamide gel electrophoresis (PAGE) the cobratoxin migrates as a 14 Kd and 8 Kd protein with a reference to comparable proteins under unreduced and reduced conditions respectively. Immunokine migrates under reduced and unreduced conditions without change. A single protein band is not obtained showing a diffuse smear from the loading gel down to a molecular weight equivalent to 8 Kd. Additionally, the protein is resistant to staining with standard coomassie dyes. By ion exchange, cobratoxin and immunokine have generally opposite properties consistent with the proteins' charges. Specialized ion-exchange chromatographic resins and conditions can be employed to confirm the retention of positive charges which are considered critical for neuroactive properties.

Detail Description Paragraph - DETX (120):

[0202] As defined by mass spectrometry the average molecular weight of immunokine is 7,933.3+.30 daltons (determined from 7 lots, 5 consecutive assays each) with a molecular weight range of 7,600 to 8,400 daltons. This molecular weight variance is expected by the nature of the reaction and ozone. As indicated above excessive ozone application can fragment the protein and insufficient levels do not modify enough amino-acid residues to render the neurotoxin atoxic. The calculated average molecular suggests the addition of 6 oxygen residues with higher molecular weights having correspondingly more. Smaller than expected molecular weights suggest protein fragmentation. Current analytical techniques allow for limited structural identification of the number and location of oxygen residues being added to the protein and rely heavily on previously published information and current chemical theory. Amino acid analyzers do not recognize unnatural amino acids and have limited capabilities for this application.

Detail Description Paragraph - DETX (136):

[0214] A TCID₅₀ of: 1000 for HIV-1.sub.Bal (CCR5-using) and 10,000 for HIV-1.sub.Lai CXCR4-using) was used to infect 10^{sup.7} PHA-stimulated peripheral blood mononuclear cells in 24 well microtiter plates. The concentrations of recombinant, ultrapure immunokine used were 1-1000 .mu.g/mL. All strains were tested in quadruplicate wells in three separate experiments. To correlate the replication endpoint concentration with a formal percent inhibitory concentration, we obtained that absolute p24 antigen content for each drug concentration. The concentration of drug that reduced the p24 antigen value of the control well by 50% (IC₅₀) was calculated using non-parametric regression analysis. Immunokine inhibited infection by HIV-1.sub.Bal by 87% compared to untreated controls and inhibited infection by HIV-1.sub.Lai by 96% compared to untreated controls with an IC₅₀ for CCR5-using isolates of 90 .eta.g/mL and an IC₅₀ of 10 .mu.g/mL for CXCR4-using isolates of HIV-1 (see figure). Immunokine did not affect proliferation as measured by [^{sup.3H}]thymidine incorporation and was not cytotoxic as determined by the soluble formazan assay.

Detail Description Paragraph - DETX (139):

[0215] Human thymus removed for cardiac procedures from children ages 4.5 months to 11 years was grown in culture up to 7 days without loss of cells. A minimum of three replicate tissue pieces were harvested for each time point or condition normally yielding 3-6 million cells per/fragments. The tissue fragments were pretreated with 100 .eta.g/mL of immunokine for 1 hour at 37.degree. C. The tissue fragments were washed in PBS, pH 7.4 and placed into sterile tubes containing 3000 TCID₅₀ of either HIV-1.sub.Bal or HIV-1.sub.Lai. The tissues were incubated at room temperature for 4 hours with gentle rocking. The tissue fragments were washed twice with PBS, pH 7.4 and transferred to 0.45 .mu.m nucleopore filters (Millipore) atop gelfoam boats (Upjohn) saturated in media [(YSSL's, 1% human serum, 50 .mu.g/ml streptomycin, 50 U/ml penicillin G, 1.times. MEM vitamin solution (GIBCO,BRL), 1.times. insulin/transferrin/sodium selenite media supplement (Sigma)], in six well plates with a maximum of 16 pieces per raft. The fragments were incubated at 37.degree. C. with 5% CO₂ for up to 3 days. At day 3, 3-4 fragments were removed and processed for flow cytometry. Quantitative evaluation of T-cell precursor subsets was performed to determine if immunokine protected thymocytes from HIV-1 induced destruction in this in vivo model. As shown in the figure, 100 .eta.g/mL of immunokine protected CD4 and CD8 single positive T-cell precursors and CD8/CD4 dual positive T-cell precursors from the HIV-1 induced destruction seen in untreated controls.

Claims Text - CLTX (2):

1. A composition for preventing HIV infection of mammalian cells, the composition comprising an anti-immunodeficiency virus immunokine capable of binding to a cellular protein in a manner that prevents HIV infection of the cell.

Claims Text - CLTX (4):

3. A composition according to claim 1 wherein the immunokine comprises an inactivated bioactive polypeptide.

Claims Text - CLTX (7):

6. A composition according to claim 1 wherein the immunokine is adapted to bind one or more of a chemokine receptor protein, and a cellular cofactor for a cellular HIV receptor protein.

Claims Text - CLTX (8):

7. A composition according to claim 6 wherein the protein to which the immunokine of the invention binds is selected from the group consisting of CD4, CXCR4 and CCR5. consisting of CD4 and CXCR4 or CCR5

Claims Text - CLTX (9):

8. A composition according to claim 3 wherein the immunokine provides a substantially native toxin structure wherein one or more of the disulfide bridges are lacking by a method selected from the ozonation of native toxin, genetic engineering, and protein synthesis.

Claims Text - CLTX (11):

10. A composition according to claim 9 wherein the immunokine comprises inactivated alpha-cobratoxin in which the disulfide bridges are substantially lacking by ozonation of native alpha-cobratoxin.

Claims Text - CLTX (12):

11. A method of inhibiting infection of a cell by HIV comprising adding to the cell an anti-immunodeficiency virus immunokine capable of binding to a cellular protein on the cell, wherein upon binding of the immunokine to the cellular protein infection of the cell by HIV is inhibited.

Claims Text - CLTX (13):

12. A method of treating HIV infection in a human comprising administering to the human an anti-immunodeficiency virus immunokine capable of binding to a cellular protein on a cell, wherein upon binding of the immunokine to the cellular protein, infection of the cell by HIV is inhibited.

Claims Text - CLTX (14):

13. A method of preparing an anti-immunodeficiency virus immunokine capable of binding to a cellular protein on a cell, the method comprising the chemical, genetic and synthetic modification of native neurotoxins.

Claims Text - CLTX (15):

14. A method of identifying a target cell for immunodeficiency virus infection, the method comprising adding to a population of cells an anti-immunodeficiency virus immunokine capable of binding to a cellular protein on a cell, wherein binding of the immunokine to a cell in the population is an indication that the cell is an immunodeficiency virus target cell.

Claims Text - CLTX (16):

15. A method of identifying a candidate anti-immunodeficiency virus compound, the method comprising isolating a test compound capable of binding to an anti-immunodeficiency virus immunokine, which immunokine binds to a cellular protein, and assessing the ability of the test compound to inhibit infection of a cell by an immunodeficiency virus in an antiviral assay, wherein inhibition of infection of the cell by the immunodeficiency virus in the presence of the test compound is an indication that the test compound is an anti-immunodeficiency virus compound.

TABLE 1. Sequences and ELISA signals of peptide phage clones affinity selected by biotinylated IgG1 b12

Clone	Peptide sequence ^a	OD ₄₀₅₋₄₉₀ ^c		
		IgG1 b12		Fab b12
		37°C	4°C	37°C
Ed1	T C LW SDL RAQ CI	ND ^b	ND	ND
B1 library	X C XX SDL XXX CI			
B1.2	G C LY SDL LAT CI	1.321	0.013	0.019
B1.11	N C LY SDL TQS CI	1.236	0.016	0.018
B1.9	N C LY SDL YAR CI	1.223	0.013	0.015
B1.20	K C MY SDL LGI CI	1.153	0.012	0.021
B1.10	D C LY SDL ESR CI	0.818	0.015	0.021
B1.4	S C LY SDL LEL CI	0.750	ND	ND
B1.3	E C MW SDL ELR CI	0.571	ND	ND
B1.12	N C LW SDL EQF CI	0.343	ND	ND
Ed2	REKRWIF SDL THT CI	ND	ND	ND
B2 library	XXXXXXX SDL XXX CI			
B2.1	HERSYMF SDL ENR CI	1.236	1.071	0.219
B2.11	CSRNLW SDL HGS CI	1.207	0.012	0.016
B2.12	NNQGCLW SDL TAS CI	1.189	0.013	0.017
B2.18	STTRCTW SDL YDS CI	1.141	0.011	0.016
B2.8	QSSSCMW SDL FQQ CI	0.992	0.016	0.018
B2.6	AQKQCTW SDL LSR CI	0.903	0.019	0.018
B2.7	RPCRGVY SDL LDK CI	0.886	0.016	0.020
B2.10	SSDHCLW SDL TMT CI	0.644	ND	ND
B2.3	LPSSCSW SDL LNR CI	0.276	ND	ND
B2.15	HTCAGTW SDL LST CI	0.252	ND	ND
gp120 ^e f88-4 ^d		1.121 0.075	0.863 0.014	1.166 0.019

^a Bold residues indicate the fixed residues in the sublibraries.^b ND experiment not done.^c Positive control.^d Negative control.^e OD₄₀₅₋₄₉₀, optical density at 405 minus 490 nm.

they elicit the cognate Abs. Our approach in developing a vaccine against HIV-1 has been to identify peptides that are specific for b12, 2F5, and 2G12 and to develop these into a vaccine that will actively target the production of broadly neutralizing Ab responses having specificities that are similar to these MAbs. This report describes the identification and characterization of a peptide that binds specifically to MAb b12.

We used biotinylated IgG1 b12 (6, 7) to screen a panel of 11 peptide libraries displayed on the major coat protein of filamentous bacteriophage (pVIII) as described in reference 4. Two clones, Ed1 and Ed2, were identified that bound b12; DNA sequencing revealed the amino-acid sequences of their displayed peptides, as shown in Table 1. The peptides displayed by these clones share the motif: SDLX₃CI; however, the Ed1 sequence bears two Cys residues whereas Ed2 bears only a single Cys, whose position is the same in both clones. Thus, a set of two phage sublibraries displaying the shared residues and reflecting the Cys content of the two Ed clones was constructed as described in reference 4. The resulting sublibraries bear the random-peptide sequences XCX₃SDLX₃CI (B1 sublibrary, two fixed Cys residues) and X₇SDLX₃CI (B2 sublibrary, one fixed Cys residue), respectively. These sublibraries

were screened with biotinylated IgG1 b12, yielding phage bearing the B1 and B2 peptide sequence families shown in Table 1. All but one of the selected phage clones bear two Cys residues, and all of the clones bound IgG1 b12, as shown by a direct phage enzyme-linked immunosorbent assay (ELISA) performed as described in reference 4.

The deduced amino acid sequences of the peptides displayed by the phage clones isolated from the sublibraries revealed a more detailed consensus for both the B1 peptides alone and the B1 and B2 peptides. Almost all of the clones selected from the B1 library contain Leu followed by an aromatic amino acid (usually Tyr) N terminal to the fixed Ser-Asp-Leu sequence. Similarly, clones from the B2 library most often bear a hydrophobic residue (usually Leu), followed by an aromatic one (usually Trp), at this site. Most of the clones from the B2 sublibrary screening have a second Cys (selected for in the screening). The peptide displayed by only one clone, B2.1, contains a single Cys residue; this peptide sequence shares similarities with those of other clones from the B2 sublibrary, and even more similarity with the Ed2 sequence, in the region N terminal to the fixed Ser-Asp-Leu sequence. The B2.1 phage was significant in binding more tightly to b12 than the other clones. Signals for almost all of the clones were strong in ELISAs performed with IgG1 b12, whereas binding was much reduced in assays using Fab b12 when it was reacted with phage at 4°C and still lower when it was reacted at 37°C (Table 1). Fab b12 bound only the B2.1 and Ed2 peptides with signals above the background, and in a side-by-side titration experiment, it was further demonstrated that the binding of b12 to B2.1 was significantly stronger than that to Ed2 (data not shown); therefore, the Ed2 peptide was not further characterized.

The ability of the B2.1 peptide to bind to the antigen-binding site of b12 was assessed in a competition ELISA. Biotinylated IgG1 b12 (1 nM) was preincubated with gp120_{Ba-L} (100 nM) and reacted with plate-adsorbed B2.1 phage. Results in Fig. 1 show that gp120 blocked the binding of IgG1 b12 to immobilized B2.1 phage, indicating that the peptide binds to the antigen-binding site of IgG1 b12. The binding to B2.1 phage was also blocked by B2.1 synthetic peptide (300 μM), nonbiotinylated IgG1 (100 nM), and the recombinant B2.1 phage but not by f88-4 phage or the unrelated synthetic peptide G45B.

The specificity of the B2.1 peptide for b12 was also assessed. MAb b12 was originally isolated from a phage-displayed Fab library constructed from the bone marrow of HIV-1-infected donor M and subsequently screened with recombinant gp120 (6). To study the specificity of the B2.1 peptide for b12, we tested whether B2.1 would select phage bearing b12 out of the repertoire of expressed Fabs from donor M. Table 2 shows that yields of 10⁻¹% were obtained after four rounds of panning of the M phage library on B2.1 phage. Moreover, the Fabs from all 12 independent phage clones that were sequenced from this phage pool were identical to b12. Thus, even though the M library contains a large number of other Fabs that recognize the CD4-binding site of gp120 (2), B2.1 selected only phage bearing Fab b12.

To produce synthetic peptides bearing the B2.1 sequence, we investigated the condition of the thiol group of the single Cys residue that is present in the B2.1 sequence. As multiple copies of the peptide-pVIII fusion protein are incorporated

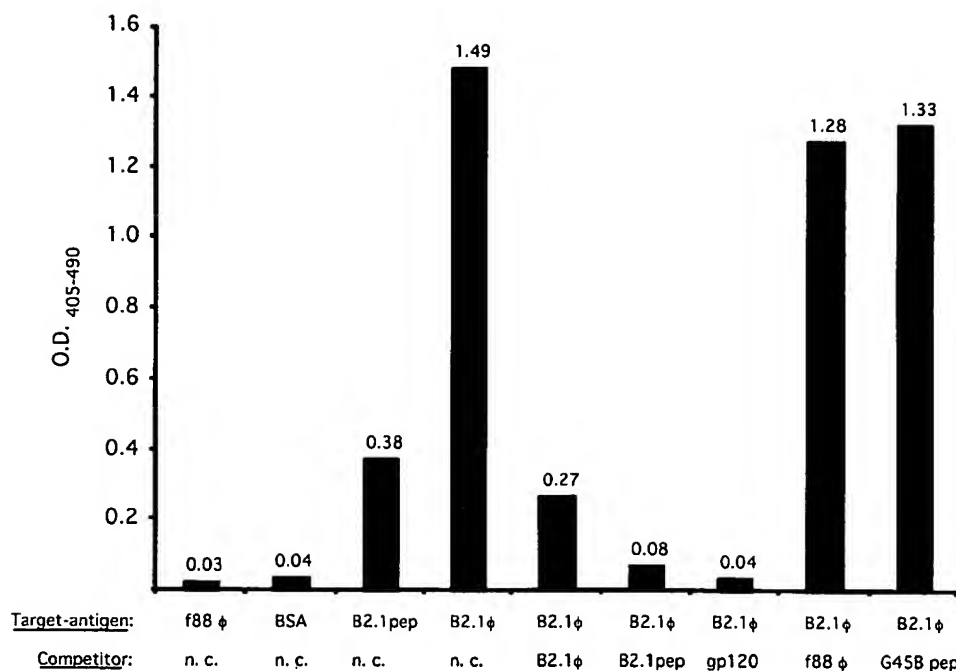


FIG. 1. Analysis of binding of biotinylated IgG1 b12 to B2.1 phage (B2.1φ) and B2.1 synthetic peptide (B2.1pep) by ELISA. Competition for IgG1 b12 binding to plate-adsorbed B2.1 phage by the following in-solution competitors is shown: 2×10^{10} B2.1 phage, 300 μM B2.1 synthetic peptide, 100 nM gp120_{Ba-L} (gp120), f88-4 phage (f88 φ), and unrelated peptide G45B, whose sequence is VERSKAFSNCYPYDVPDYASLRS. BSA is bovine serum albumin, and n. c. indicates no in-solution competitor. O.D.₄₀₅₋₄₉₀, optical density at 405 minus 490 nm.

into the phage coat, the single Cys residue of B2.1-pVIII may potentially be in a reduced form (as a reduced thiol group) or disulfide bridged to a second copy of the B2.1-pVIII fusion protein. If the B2.1 peptide-pVIII fusion protein existed as a homodimer on the phage surface, it would have roughly twice

TABLE 2. Percent yields of four successive rounds of affinity selection of phage-displayed Fab library M with B2.1 phage^a

Round and phage or protein immobilized on plate	Input (TU, 10 ⁹)	Output (TU, 10 ⁴)	% Yield
1			
B2.1	62	4.8	9.2×10^{-5}
f88-4	62	4.0	7.8×10^{-5}
gp120	62	9.6	1.8×10^{-4}
2			
B2.1	6.6	2.4	3.6×10^{-4}
f88-4	6.6	1.6	2.4×10^{-4}
gp120	6.6	140	2.1×10^{-2}
3			
B2.1	2.1	14	6.8×10^{-3}
f88-4	2.1	14	6.8×10^{-3}
gp120	2.1	3,200	1.5
4			
B2.1	2.7	400	1.5×10^{-1}
f88-4	2.7	19	8.8×10^{-3}
gp120	2.7	110	4.1×10^{-1}

^a For panning, 400 ng of gp120_{SF2} and 5×10^{10} recombinant B2.1 or f88-4 phage were immobilized on a plate. Input and output phage values are given in ampicillin-resistant transfecting units (TU).

the molecular weight of the pVIII monomer. Thus, B2.1 phage were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) using Tris-Tricine buffer as previously described (35). Phage samples were initially treated with the thiol-reactive reagent *N*-ethylmaleimide (NEM) (Fig. 2 A), which blocks free thiols that might be present on the phage coat and would prevent the formation of pVIII dimers after solubilization of the phage coat proteins with heat and SDS. Hence, if B2.1-pVIII fusions bear free thiols and are monomeric, reaction with NEM should prevent them from dimerizing after dissociation of the phage. Alternatively, if the B2.1-pVIII fusions exist on the phage coat as dimers (produced by disulfide bridging between displayed B2.1 peptides), treatment of the phage with NEM, followed by boiling in the presence of SDS, should not affect their migration as dimers. The results shown in Fig. 2A reveal that the recombinant pVIII from B2.1 phage migrates as a dimer that is not affected by NEM treatment, whereas it migrated as a monomer in samples treated with the reducing agent dithiothreitol (DTT). Samples sequentially treated with NEM and DTT also behaved as monomers. This proves that most or all of the B2.1 peptide displayed on the phage surface is homodimeric.

In contrast to the B2.1 dimers, the clones that display peptides containing two Cys residues produced monomers or, less often, a mixture of monomers and dimers, with monomers predominating (data not shown). This result suggests that, as opposed to B2.1, these clones bear mostly intrachain disulfide bridges, consistent with the results of Zwick et al. (35). Their survey of phage-displayed peptides bearing one and two Cys residues showed that almost all containing two Cys residues

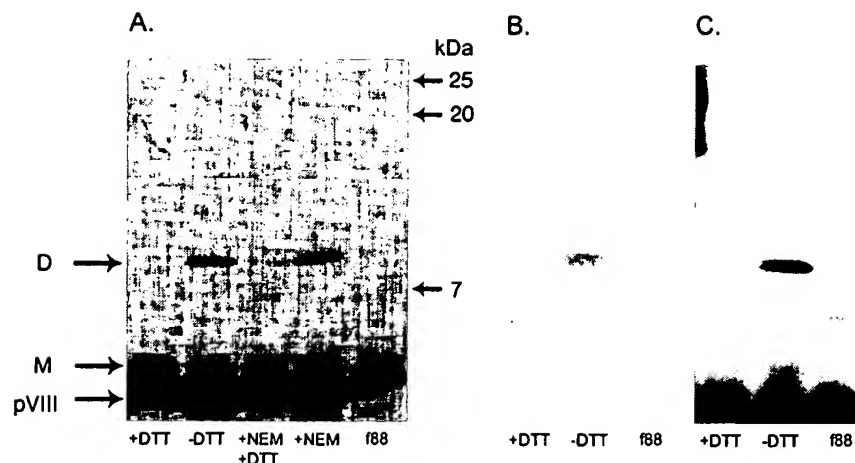


FIG. 2. SDS-PAGE analysis of the f88-4 wild-type phage (f88) and recombinant B2.1 phage (all others). Phage were left untreated or treated with DTT, NEM, or NEM followed by DTT and then analyzed by SDS-PAGE. Monomeric (M) and dimeric (D) recombinant pVIII proteins are shown. Proteins in similar gels were either silver stained or transferred to a membrane and subjected to Western blotting with anti-phage Ab or IgG1 b12. Panels: A, silver-stained gel; B, Western blot using IgG1 b12 to show the reactive dimer; C, Western blot using rabbit anti-phage Ab to show the wild-type and recombinant pVIII proteins.

are cyclic whereas all of those bearing a single Cys residue form homodimers.

The requirement for an intact disulfide bridge for the antigenicity of B2.1, and of clones bearing cyclic peptides, was assessed by Western blot experiments (15) using IgG1 b12 or a rabbit polyclonal anti-phage Ab for detection. Figure 2B shows that IgG1 b12 binds only to the B2.1-pVIII fusion in its dimeric form. Staining with IgG1 b12 was present at the site of the dimer but not at the monomer, whereas both forms were detected by the anti-phage Ab (Fig. 2C). A clone selected from the B1 sublibrary, bearing a peptide-pVIII fusion containing two Cys residues, was also tested by a Western blot assay with IgG1 b12. It produced a band much weaker than that of the B2.1 phage, whereas blotting with the anti-phage Ab produced a recombinant band with an intensity similar to that of the B2.1 clone (data not shown). This supports the conclusions drawn from the ELISA data (Table 1) indicating that IgG1 b12 does not bind as tightly to peptides containing two Cys residues as it does to the B2.1 homodimer. Moreover, as with the B2.1 homodimer, reduction by DTT of the intrachain disulfide bridge of clones containing peptides bearing two Cys residues ablated

b12 binding in the ELISA; thus, disulfide-bridging is also required for their antigenicity (data not shown).

The location of the Cys residue (and hence the disulfide bridge) in the B2.1 sequence is crucial to its reactivity with b12. Phage bearing mutations in the B2.1 peptide sequence were prepared and assayed for the abilities to bind IgG1 b12 and produce homodimer and/or monomer bands on analysis by SDS-PAGE. As shown in Table 3, replacement of Cys₁₄ with Ser ablated dimer formation and Ab binding. Interestingly, replacement of Ser₄ with Cys ablated binding, regardless of whether the residue at position 14 was Cys; even the dimeric form of this mutant peptide did not bind b12 significantly. Thus, the antigenicity of B2.1 is strongly affected by the presence and location of the disulfide bridge that produces homodimers.

To study the affinity of the B2.1 homodimer out of the context of the phage coat, a synthetic version of the B2.1 peptide was prepared as a disulfide-bridged homodimer with the sequence NH₃-HERSYMFSDELNRCIAAEGK-NH₂ (Multiple Peptide Systems, San Diego, Calif.; monomer molecular weight, 2,354.6; >95% pure and >95% dimer). This

TABLE 3. Binding of b12 IgG to B2.1 phage mutants

Phage clone	Peptide sequence ^a	IgG1 b12 ^a		SDS-PAGE ^b		Western blot ^c b12 binding
		3 nM	30 nM	Dimer	Monomer	
B2.1	HERSYMFSDELNRCI	1.00	1.04	+	+	+
B2.1-Δ Cys	HERSYMFSDELNRSI	0.02	0.04	-	+	-
B2.1-5' Cys	HERCYMFSDELNRSI	0.02	0.05	+	+	-
B2.1-CC	HERCYMFSDELNRCI	0.03	0.13	-	+	-
f88-4		0.02	0.03	-	-	-
None		0.02	0.03	NA ^d	NA	NA

^a Values are optical densities at 405 minus 490 nm from a direct phage ELISA.

^b Wild-type and mutant B2.1 phage were subjected to SDS-PAGE in the presence or absence of DTT; the dimer and monomer columns show the results for nontreated and DTT-treated phage, respectively. Symbols: + detection of recombinant B2.1-pVIII fusion band on silver-stained gels; - no band observed.

^c A plus sign indicates reactivity with IgG1 b12 in the Western blot, and a minus sign indicates no reactivity.

^d NA, not applicable.

^e Bold residues indicate sites at which amino acid replacements were made based on the B2.1 clone sequence.

TABLE 4. Reconstruction panning of Fab b12 phage versus B2.1 peptide, B2.1 phage, and gp120 Ba-L^a

Phage reconstruction		B2.1 peptide	B2.1 phage	gp120 Ba-L
Fab b12	DP47/AD27			
10 ¹⁰		2.7 × 10 ⁻³	2.1 × 10 ⁻¹	3.2 × 10 ⁻¹
10 ⁹	9 × 10 ⁹	3.0 × 10 ⁻³	1.4 × 10 ⁻¹	2.0 × 10 ⁻¹
10 ⁸	10 ¹⁰	1.5 × 10 ⁻⁴	7.7 × 10 ⁻²	2.8 × 10 ⁻²
10 ⁷	10 ¹⁰	1.6 × 10 ⁻⁴	3.1 × 10 ⁻⁴	1.0 × 10 ⁻³
10 ⁶	10 ¹⁰	4.5 × 10 ⁻⁴	5.8 × 10 ⁻⁵	2.7 × 10 ⁻⁴
10 ⁵	10 ¹⁰	1.2 × 10 ⁻⁴	1.3 × 10 ⁻⁴	1.2 × 10 ⁻⁴
10 ⁴	10 ¹⁰	3.4 × 10 ⁻⁴	2.7 × 10 ⁻⁴	3.1 × 10 ⁻⁴
	10 ¹⁰	1.6 × 10 ⁻⁴	2.0 × 10 ⁻⁴	4.9 × 10 ⁻⁴

^a Decreasing amounts of Fab b12 phage were mixed with DP47/AD27 phage to a total of 10¹⁰ particles and screened in one single round with the three antigens. Results are expressed as percent yields of ampicillin-resistant transfecting units.

synthetic B2.1 peptide was used as a target with which to isolate phage bearing b12 Fab from the M library; but no phage were selected (data not shown), indicating that the synthetic peptide does not bind b12 as tightly as the phage-borne one. To verify the relatively weak interaction of the synthetic peptide with b12 compared to phage-borne B2.1, a panning reconstruction experiment was performed in which phage bearing Fab b12 were mixed with various amounts of phage bearing unrelated Fab AD27/A47 (as a background control phage). The Fab phage were panned side by side in wells coated with gp120, B2.1 phage, or B2.1 peptide. The results in Table 4 show that gp120 and B2.1 phage enriched b12 phage 50- to 100-fold better than did the synthetic B2.1 peptide. Thus, the affinity of the phage-borne B2.1 for the b12 Fab appears to be stronger than that of the synthetic peptide.

We also prepared a biotinylated, synthetic version of the B2.1 peptide having the sequence NH₂-HERSYMFSLENR CIAAE-Orn(biotin)-KK-NH₂ (Multiple Peptide Systems; monomer molecular weight, 2,767.6; >95% pure and 80% dimer). This peptide (bio-B2.1) was biotinylated so that it could be bound to immobilized streptavidin in ELISA wells and directly detected during the production of conjugates for immunization, regardless of its IgG1 b12 antigenicity. The relative affinity of MAb b12 for the B2.1 sequence presented in different forms was assessed by direct titrations using Fab and IgG1 b12. The titrations were performed on streptavidin-captured and plate-immobilized bio-B2.1 peptide, as well as with recombinant B2.1 phage (and gp120 as a positive control). Figure 3A shows that the binding of Fab b12 to both plate-immobilized and streptavidin-captured synthetic B2.1 peptide was almost undetectable over the background. In contrast, Fab binding to recombinant B2.1 phage was strong and followed a titration curve similar to that of gp120 (Fig. 3A), suggesting that the affinities of b12 for gp120 and phage-displayed peptide are similar (K_{ds} of 3 nM [gp120_{MN}] and 9.1 nM [gp120_{LAI}] have been reported by Roben et al. [31] and Parren et al. [28], respectively). Although the results were somewhat different when IgG1 b12 was used instead of Fab for the titration ELISA (which was most likely due to the inherent avidity of the IgG), a similar trend was observed (Fig. 3B). IgG1 b12 reacted with both phage-displayed and synthetic B2.1 peptide; however, it bound more tightly to recombinant B2.1 phage than to either form of the synthetic peptide. Moreover, the Ab showed better

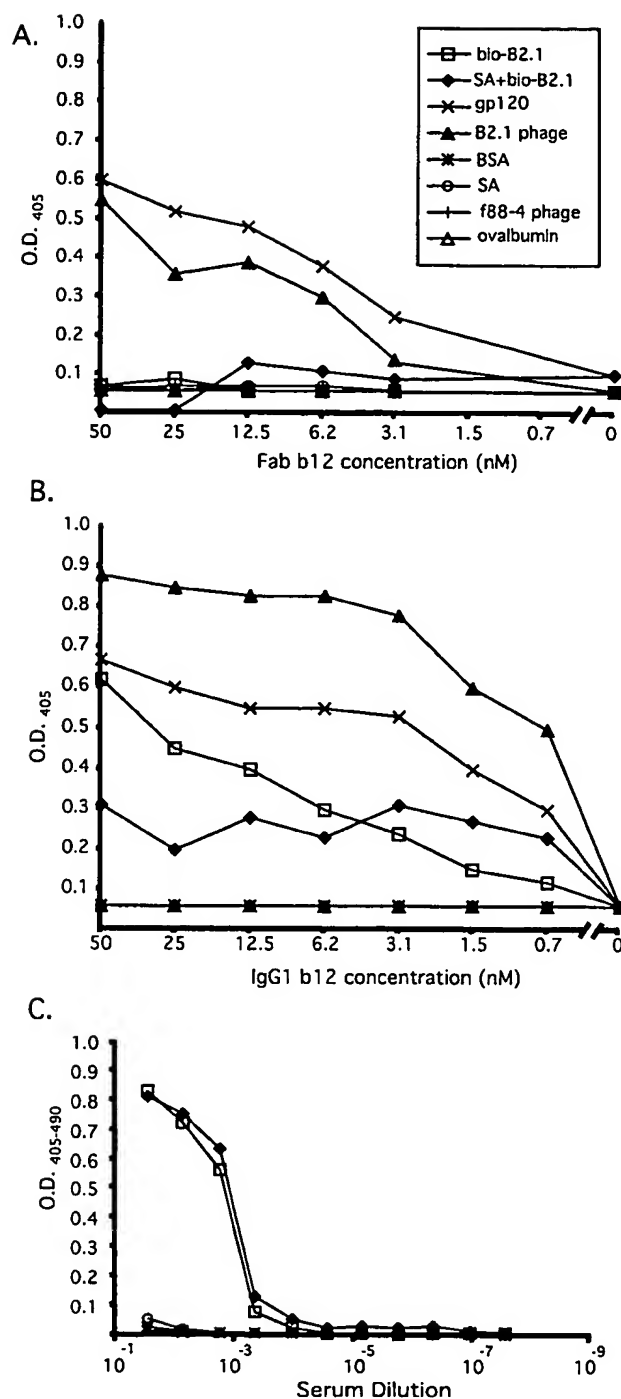


FIG. 3. Titration of Fab b12 (A), IgG1 b12 (B), and murine anti-B2.1 peptide serum (C) on different immobilized antigens. Twofold dilutions of Fab and IgG1 b12 and fourfold mouse serum dilutions were reacted with biotinylated B2.1 directly adsorbed to ELISA wells (bio-B2.1), biotinylated B2.1 bound to immobilized streptavidin (SA+bio-B2.1), gp120_{Ba-L}, B2.1 recombinant phage, f88-4 phage, bovine serum albumin (BSA), ovalbumin, and streptavidin (SA). O.D., optical density.

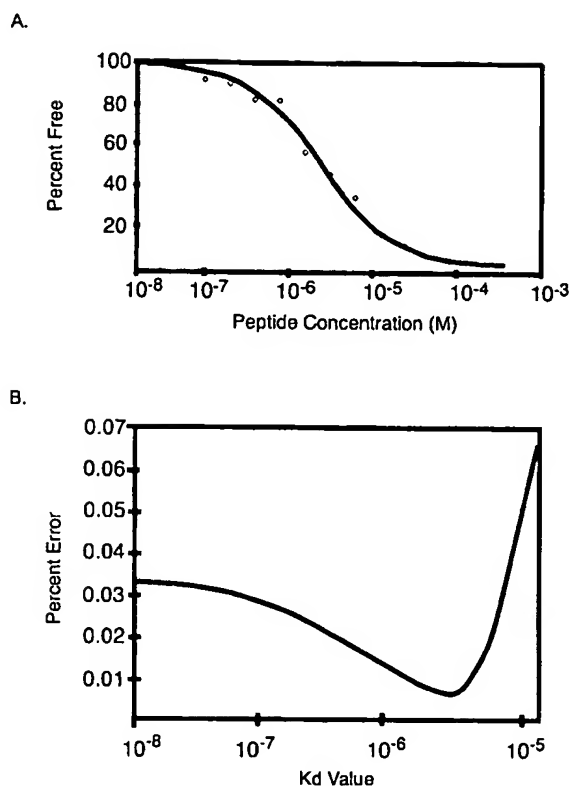


FIG. 4. Kinetics of binding of IgG1 b12 to B2.1 peptide in-solution. (A) Percent free Ab versus molar concentration of peptide; data (diamonds) and the best-fit theoretical curve are shown. (B) Percent error from fit of the data in Fig. 3A to the best-fit curves calculated for a range of K_d 's. The 95% confidence interval calculated for this experiment is 1.3 to 3.7 μ M.

binding to the plate-adsorbed peptide than to the streptavidin-captured one; thus, it was able to discriminate between these two means of presenting the peptide. In contrast to IgG1 b12, the IgG from a mouse that had been immunized with a B2.1 conjugate vaccine (see below) showed no discrimination between the streptavidin-bound and plate-adsorbed forms of bio-B2.1 (Fig. 3C) and binding to gp120 was undetectable. These results indicate that the ability of b12 to discriminate between plate-immobilized peptide and streptavidin-captured peptide is linked with its capacity to bind gp120, again suggesting that a specific B2.1 structure (or set of structures) is responsible for its antigenicity for b12. It is apparent from these titration experiments that the most antigenic structure of the B2.1 sequence is best represented by the recombinant peptide in the context of the phage coat.

To assess the range of affinities of the different peptides for b12, the in-solution binding affinity of IgG1 b12 for the B2.1 peptide was determined by using a KinExA 3000 (Kinetic Exclusion Assay) instrument (Sapidyne Instruments, Inc., Boise, Idaho) (3) as described in reference 10. KinExA measurements involving in-solution monovalent antigen yields affinity constants that are independent of the Ab valency. The data in Fig. 4 show that the interaction between IgG1 b12 and the free peptide closely follow a 2.5- μ M K_d best-fit theoretical curve derived from a simple second-order kinetic model (Fig. 4A).

Comparison of the percent root mean square deviation errors (Fig. 4B) produced from the fit of these data to the best-fit curves calculated for a range of K_d 's revealed the accuracy of the K_d found for b12 and B2.1 in solution. This K_d is \approx 200-fold higher than the 9.1-nM K_d measured for the interaction between Fab b12 and recombinant gp120 from HIV-1_{LAI}, as determined by surface plasmon resonance (31). However, the K_d is lower than the \approx 100 μ M found for a synthetic cyclic peptide made from one of the clones isolated from the B1 library by competition ELISA of that peptide with Fab b12 (A. Satterthwait and J. K. Scott, unpublished data).

The Fab and IgG1 titration data and the in-solution affinities of b12 for B2.1 and gp120 may be used to provide very rough reference values from which the affinity of the plate-bound and phage-displayed peptides could be interpolated. Given the range of 9 nM for gp120_{LAI} and \approx 3 μ M for the free peptide (and assuming that the affinity of free B2.1 is similar to that of bio-B2.1 captured on streptavidin), we speculate that the plate-adsorbed peptide binds with a K_d ranging between 20 and 500 nM. The phage-displayed recombinant peptide shows the highest affinity for binding to Fab b12, with the data suggesting a K_d value close to that of b12 for gp120.

Taken together, our results support the idea that the affinity of the B2.1-b12 interaction is dependent on the environment in which the peptide is presented to b12. The data suggest the existence of different structures of the B2.1 homodimer and indicate that the predominant structure of B2.1 in solution (and tethered, via biotin, to streptavidin) is either unfolded or unstable and different from the one(s) that it assumes in the context of the phage coat. Our results obtained with synthetic and recombinant B2.1 peptides indicate that the structure of the homodimer could be further optimized to maximize its antigenicity. B2.1 binds b12 preferentially when fused to the pVIII coat protein and displayed on the phage surface, perhaps because the highly structured phage coat provides a more rigid and/or stable environment for the peptide. This would be in keeping with the work of Jelinek et al. (16), which shows that antigenic peptides fused to pVIII produce nuclear magnetic resonance (NMR)-definable structures, even though the free peptide in solution does not.

The sequences of the peptides we have discovered (Table 1), especially B2.1, show significant homology to the D-loop region of gp120 (residues 273 to 285). The residues of the B2.1 sequence that are shared with D-loop sequences from a number of gp120s are bolded in HERSYMFSDLENRCI. The D loop region of gp120 contains a number of residues that are highly conserved among HIV-1 isolates from different clades. Frequencies in gp120s from all clades for the bolded residues in the B2.1 sequence are 96% for Arg273, 96% for Ser274, 52% for Phe277, 99% for Ser or Thr at position 278, 99% for Asp or Asn at position 279, and 98% for Ile285 (frequency data were taken from the Los Alamos Env sequence database at: <http://hiv-web.lanl.gov/>). As well, residue Asp279 of the D loop also makes contact with CD4 and thus forms part of the CD4 binding site on gp120 (16a).

Figure 5 shows the sequence and structure of the D loop of HXB2 gp120; it appears to be partially stabilized by the interaction between the side chains of Val275 and Ile284. The b12-selected clones that contain two Cys residues most often have loop lengths of eight residues (Table 1). If the sequences

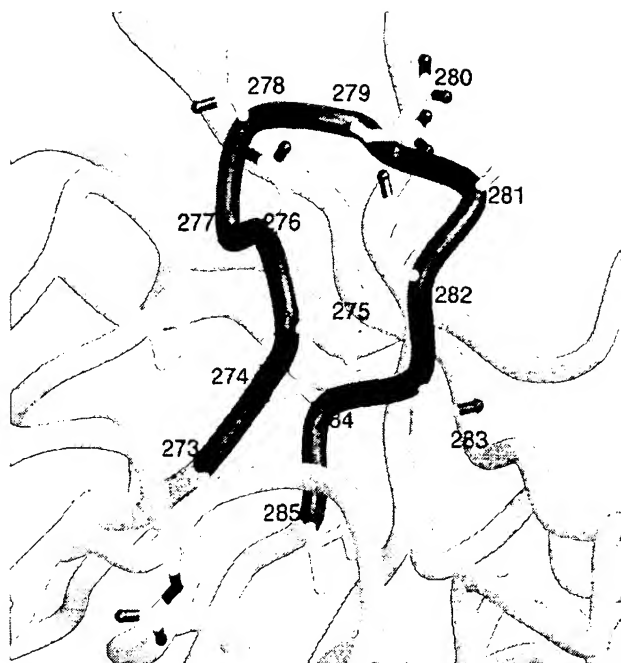


FIG. 5. Structure of the D loop of gp120, residues 273 to 285, taken from the HXB2 HIV-1 isolate. The sequence of this region, whose alpha-carbon backbone is shown in red, is RSVNFTD NAKTII. Residues shared with the B2.1 peptide, HERSYMFS DLENRCI, are in bold type.

of the cyclic peptides having 8-mer loops are overlaid on the D loop, with their N- and C-terminal Cys residues being placed where Val275 and Ile284 of the D loop are located, respectively, homologous residues shared between the two are perfectly aligned. The location of the FSD sequence aligns with that of the D loop's FTD sequence, and the N-terminal Ile of the peptides aligns with the gp120 Ile285.

The homologies between residues in B2.1 and conserved residues in the D loop, as well as the structural homologies with the cyclic peptides, lead us to predict that the D loop of gp120 is involved in binding the b12 Ab, with the residues RS, FSD, and I being of importance in maintaining a D-loop-like structure and/or in making direct contacts with the b12 Ab. The crystallographic structure of IgG1 b12 has been elucidated, both in the free form and bound to the B2.1 peptide (E. O. Saphire, personal communication). These structures should prove useful in directing further optimization of the B2.1 peptide as a b12 ligand and as a gp120 mimic and in characterizing the gp120 epitope for b12 at the atomic level.

Our goal in developing a peptide mimic of the b12 epitope is to use it in a vaccine against HIV-1 infection to elicit b12-like neutralizing Abs. We conjugated the biotinylated synthetic peptide to wild-type phage and ovalbumin using BS3 as a cross-linker. The conjugates bound IgG1 b12, as shown by Western blot assay, ELISA, and immunoprecipitation, indicating that the antigenic structure of B2.1 was conserved after the conjugation. We found that both conjugates were immunogenic in mice and rabbits but did not elicit significant gp120-cross-reactive Ab titers, indicating that b12-like Abs were not produced at detectable levels (data not shown). At least two

reasons may account for this: (i) the relatively low affinity of b12 for the synthetic version of the peptide and/or (ii) the species barrier (b12 has an 18-residue-long H3, and Abs with such features are not produced in mice). We also carried out immunizations with B2.1 recombinant phage, which produced only moderate anti-peptide Ab titers, accompanied by high anti-phage Ab responses. In addition to the species problem mentioned above, we believe that the relatively low copy number of the B2.1 homodimer displayed on the phage surface (≈ 200 copies/phage) explains this result.

Thus, the isolation and characterization of the B2.1 peptide constitute the first stage of a new strategy for targeting the production of Abs against a single prespecified neutralizing epitope on HIV-1. However, the generation of a successful B2.1 immunogen requires further optimization at several levels. Our results indicate that the structure of the homodimer displayed on the phage coat is best for the binding of b12; thus, the recombinant phage is the primary target of our efforts at structure-based optimization of B2.1 as an antigen and immunogen. We are currently assessing the peptide residues that are critical for b12 binding in the context of the phage. These functional data, coupled with the crystallographic data mentioned above, should provide insight into further optimization of the B2.1 peptide. Such optimization also requires knowledge of the phage-borne structure of B2.1. Thus, we are also making efforts to raise the copy number of the B2.1 homodimer on the phage coat, to allow NMR-based analyses, and to generate soluble B2.1 fusion proteins so that the dimer can be studied in a "monovalent" protein format (compared to phage particles, which bear multiple copies of the dimer). Other immunization strategies (a prime-boost approach) and species (monkeys and XenoMouse) will also be explored.

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Molecular Features of the Broadly Neutralizing Immunoglobulin G1 b12 Required for Recognition of Human Immunodeficiency Virus Type 1 gp120

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IgG1 b12 is a broadly neutralizing antibody against human immunodeficiency virus type 1 (HIV-1). The epitope recognized by b12 overlaps the CD4 receptor-binding site (CD4bs) on gp120 and has been a target for vaccine design. Determination of the three-dimensional structure of immunoglobulin G1 (IgG1) b12 allowed modeling of the b12-gp120 interaction in which the protruding third complementarity-determining region (CDR) of the heavy chain (H3) was crucial for antibody binding. In the present study, extensive mutational analysis of the antigen-binding site of Fab b12 was carried out to investigate the validity of the model and to identify residues important for gp120 recognition and, by inference, key to the anti-HIV-1 activity of IgG1 b12. In all, 50 mutations were tested: 40 in H3, 4 each in H2 and L1, and 2 in L3. The results suggest that the interaction of gp120 with H3 of b12 is crucially dependent not only on a Trp residue at the apex of the H3 loop but also on a number of residues at the base of the loop. The arrangement of these residues, including aromatic side chains and side chains that hydrogen bond across the base of the loop, may rigidify H3 for penetration of the recessed CD4-binding cavity. The results further emphasize the importance to gp120 binding of a Tyr residue at the apex of the H2 loop that forms a second finger-like structure and a number of Arg residues in L1 that form a positively charged, shelf-like structure. In general, the data are consistent with the b12-gp120 interaction model previously proposed. At the gene level, somatic mutation is seen to be crucial for the generation of many of the structural features described. The Fab b12 mutants were also tested against the b12 epitope-mimic peptide B2.1, and the reactivity profile had many similarities but also significant differences from that observed for gp120. The paratope map of b12 may facilitate the design of molecules that are able to elicit b12-like activities.

It is of fundamental importance to the global human immunodeficiency virus type 1 (HIV-1) vaccine effort to look for potential ways in which to elicit an effective neutralizing antibody response against HIV-1 (6, 8, 10, 27, 32, 53, 68, 88, 92). The target of neutralizing antibodies against HIV-1 is the envelope spike, which consists of the surface glycoprotein gp120 and the transmembrane protein gp41. Although not formally proven, it is generally accepted that the spike is a trimer of gp120-gp41 heterodimers (12, 13, 33, 40, 60, 83, 87). One of the consequences of this quaternary arrangement is that a number of conserved epitopes that are well exposed on purified, monomeric gp120 and gp41 are buried or partially buried in the trimeric gp120-gp120, gp41-gp41, or gp120-gp41 interfaces within the native spike (29, 62, 67, 69, 86). The relative inaccessibility of conserved epitopes in the trimeric spike likely explains the paucity of neutralizing monoclonal antibodies against HIV-1 (8) as well as the low titers of isolate cross-neutralizing antibodies typically found in the serum of animals or humans immunized with soluble envelope protein (15, 20,

21, 23, 26, 45, 72, 81, 85) or even during natural infection with HIV-1 (38, 48).

Nevertheless, a few broadly neutralizing antibodies against HIV-1 have been described. Immunoglobulin G1 (IgG1) b12 binds to the CD4 receptor binding site (CD4bs) on gp120 (4, 9, 11, 62), 2G12 binds to a carbohydrate-rich epitope on the silent face of gp120 (63, 70, 80), and 2F5 binds to a linear epitope close to the membrane on the ectodomain of gp41 (52, 61, 94). In addition, three novel antibodies have recently been identified as having broad neutralizing activity: Fab X5, which binds an epitope on gp120, the exposure of which is enhanced by CD4 binding (51), 4E10 (74, 94), and Z13 (94), which bind immediately C-terminal to 2F5 on gp41. These antibodies stand out among the population of known human antibodies as being relatively potent and able to neutralize a wide range of primary isolates of HIV-1 (21, 51, 94) and in combination have been shown to neutralize HIV-1 with some degree of synergy (43, 95). Moreover, IgG1 b12 has recently been shown to be effective at neutralizing primary isolates of subtype C, which is responsible for the greatest number of infections worldwide (7). Importantly, IgG1 b12 is able to completely protect macaques against vaginal challenge with the simian immunodeficiency virus-HIV hybrid SHIV_{162P4} (58). This study, together with other passive antibody protection studies (2, 14, 44, 46, 56, 73), establishes parameters by which antibody can mediate

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sterile protection against retroviral challenge and illustrates the potential of broadly neutralizing antibodies for controlling HIV-1, at least in animal models.

Of the panel of broadly neutralizing anti-HIV-1 monoclonal antibodies, IgG1 b12 is the best characterized at the molecular level. Somatic variants of b12 were available essentially since its discovery by phage display methodology, providing an early indication of residues in the antibody variable regions that influence binding activity (4, 9, 57). Later, *in vitro* selection experiments were performed in which a complementarity determining region (CDR) walking strategy was used to identify variants of b12 with greater affinity for monomeric gp120 and, in some cases, with an enhanced ability to neutralize HIV-1 (5, 90). Recently, the three-dimensional structure of the whole IgG1 b12 molecule was determined (66), providing a structural framework for attempts to elucidate its broadly neutralizing activity. In particular, a Trp residue displayed at the apex of a long protruding H3 loop may allow b12 to penetrate and fill the hydrophobic cavity of the CD4bs on gp120 in a way analogous to Phe43 of CD4 (66). From a docking model of b12 with the core of gp120 (39), b12 also makes contacts on the inside face of the V1/V2 loop stem of gp120 and with the D loop of gp120 by a canyon created by CDRs H3, L1, and L3 (66). This model can now be tested by additional structural and functional studies.

IgG1 b12 is also arguably the best characterized of a group of antibodies known as anti-CD4bs antibodies, which compete with CD4 and with each other in binding to gp120. Many human anti-CD4bs antibodies (besides IgG1 b12) have been described by various groups (49, 50, 75), including 15e (30), F105 (76), F91 (50), 1125H (77), 21h (30, 75), 654-30D (41), and Fab b6 (57, 62), the last of which was isolated from the same seropositive subject from whom b12 was cloned. These anti-CD4bs antibodies often show broad reactivity with monomeric gp120s from different isolates of HIV-1 but do not, however, show the neutralizing activity of b12 (21, 22, 62). The difference has been associated with the ability of b12 but not of other anti-CD4bs antibodies to bind well to the trimeric envelope spike on the surface of virions (59). It would seem that b12 is able to bind with reasonably high affinity to both monomeric and trimeric forms of gp120, whereas the other CD4bs antibodies bind well only to the monomeric form.

Clearly, one would like to have immunogens capable of eliciting b12-like antibodies. To this end, we have been exploring the interaction of b12 with gp120 from a number of aspects. These include determination of the crystal structure of b12 (66), docking of b12 with the structure of the core of gp120 (66), and examination of the effects of mutations of gp120 residues on the b12-gp120 interaction (55). Here, we approached the problem from the point of view of the antibody by attempting to identify the key structural features of b12 required for gp120 binding through extensive mutagenesis of b12 residues. At the same time, for comparative purposes, we looked at the effects of mutations on the interaction of b12 with a peptide mimotope, B2.1, that binds b12 specifically and is being studied as a vaccine lead (93). The results provide functional data relevant to the docking model presented previously (66) and reveal specific requirements at the tip and base of the CDR H3 finger of b12 for gp120 recognition. In addition, a cluster of arginine residues in the CDR L1 region

forming a shelf-like structure and a prominently displayed Tyr residue in CDR H2 are shown to be crucial. The potential implications for eliciting b12-like antibodies by vaccination are discussed in terms of the demands that this puts on the antibody repertoire.

MATERIALS AND METHODS

Mutagenesis and crude Fab preparation. b12 Fab mutants were engineered with the QuikChange mutagenesis kit (Stratagene) according to the manufacturer's directions with pComb3H vector DNA, encoding wild-type b12 Fab, as the template. A similar approach was used to engineer the Fab b6 mutants. The sequences of the mutant clones were verified by DNA sequencing within the variable regions. A complete list of the Fab b12 mutants engineered in this study is included in Fig. 1. The CDRs were defined with IMGT delimitations (<http://imgt.cnusc.fr:8104/home.html>) (42) except for H3, for which conserved residues A93 and R94 were omitted, as per the Kabat and Wu definition (34).

The preparation of crude Fab supernatants has been described previously (3). Briefly, the mutant clones, wild-type b12, and an irrelevant Fab negative control were transformed separately into *Escherichia coli* XL1-Blue cells (Stratagene), and single colonies were used to inoculate 10-ml cultures in SB medium containing 50 µg of carbenicillin and 10 µg of tetracycline per ml. The cultures were shaken at 300 rpm at 37°C for 6 to 8 h, then induced with 1 mM isopropylthiogalactopyranoside (IPTG), and incubated overnight at 30°C with shaking. The next day, the cultures were centrifuged at 5,000 × g for 15 min at 4°C, the pellets were resuspended in 1 ml of phosphate-buffered saline (PBS, pH 7.0), and the bacterial suspensions were subjected to four rounds of freeze-thawing. The bacterial debris was pelleted at 14,000 rpm in a microcentrifuge, and the supernatants were supplemented with bovine serum albumin (BSA) and Tween 20 (1% and 0.025% final concentrations, respectively). Duplicate or triplicate crude Fab supernatants were prepared to lessen the effect of culture-to-culture variation in Fab production, pooled, and used directly for enzyme-linked immunosorbent assays (ELISAs) as described below.

Crude Fab ELISA. Ninety-six-well plates (one-half diameter, flat-bottomed; Costar) were coated with 50 µl of PBS containing 50 ng of goat anti-human IgG F(ab')₂ (Pierce), 75 ng of gp120_{RFL} (Progenics), 50 ng of oligomeric gp120_{H1H3} (ImmunoDiagnostics, Inc.), or 7 × 10⁹ B2.1 phage particles (the B2.1 peptide HERSYMFSDLENRCI is a disulfide-bridged, homodimeric peptide displayed as a fusion to the N terminus of pVIII on the filamentous phage [93]) and incubated overnight at 4°C. The wells were washed twice with PBS containing 0.05% Tween 20 and blocked with 3% BSA at 37°C for 1 h. The wells were washed once, and 50 µl of the bacterial supernatants containing Fab diluted in PBS containing 1% BSA and 0.025% Tween 20 was added. The plates were incubated for 2 h at 37°C, the wells were washed four times, goat anti-human Fab conjugated to alkaline phosphatase (Pierce), diluted 1:500 in PBS containing 1% BSA and 0.025% Tween 20, was added to the wells, and the plate was incubated at room temperature for 30 min. The wells were washed five times and developed by adding 50 µl of alkaline phosphatase substrate, prepared by adding one tablet of disodium *p*-nitrophenyl phosphate (Sigma) to 5 ml of alkaline phosphatase staining buffer (pH 9.8), according to the manufacturer's instructions.

After ≈30 min, the optical density at 405 nm was read on a microplate reader (Molecular Devices). The concentration of Fab was determined with the anti-Fab ELISA (full curve, threefold dilution series) with simple linear regression; the concentrations of Fab in the samples were usually within about twofold of that of wild-type Fab b12 except for mutants V95A, Y53G, 3D3A, and 3D3N, which were consistently 4- to 10-fold less abundant. A full threefold ELISA binding curve was also generated for groups of Fab mutants alongside wild-type b12 and a negative Fab control against gp120 or B2.1 phage. Apparent affinities were calculated as the antibody concentration at half-maximal binding. Apparent affinities as a percentage of that of wild-type Fab b12 were calculated with the formula [(apparent affinity of the wild type)/(apparent affinity of the mutant)] × 100. All samples were tested at least twice, and the mean was taken as the final reported value.

Competition ELISA with purified Fab. Ninety-six-well plates were coated with gp120, washed, and blocked, as above. Wild-type and representative mutants of Fab b12 were purified as described previously (3) with protein G-Sepharose columns (Fast Flow; Pharmacia) and verified to be >90% pure by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The purified Fabs were added to the wells at various concentrations in the presence of a single concentration of biotinylated Fab b12 that was previously determined to generate an ELISA signal of 50 to 75% of maximal. After 2 h of incubation at 37°C, the plate was washed, and a streptavidin-horseradish peroxidase conjugate (Jack-

A	Cl one ID	CDR H3
	b12 (w.t.)	V G P Y S W D D S P Q D N Y Y M D V
	Solvent access#	0 0 3 2 2 4 3 1 3 3 1 2 0 1 0 0 1 1
	V95A	A - - - - - - - - - - - - - - - - -
	V95I	I - - - - - - - - - - - - - - - - -
	G96A	- A - - - - - - - - - - - - - - - - -
	P97A	- - A - - - - - - - - - - - - - - - -
	P97E	- - E - - - - - - - - - - - - - - - -
	Y98A	- - - A - - - - - - - - - - - - - - -
	Y98W	- - - W - - - - - - - - - - - - - - -
	S99A	- - - - A - - - - - - - - - - - - - -
	S99G	- - - - G - - - - - - - - - - - - - -
	W100A	- - - - - A - - - - - - - - - - - - -
	W100F	- - - - - F - - - - - - - - - - - - -
	W100S	- - - - - S - - - - - - - - - - - - -
	W100V	- - - - - V - - - - - - - - - - - - -
	D100aA	- - - - - A - - - - - - - - - - - - -
	D100aE	- - - - - E - - - - - - - - - - - - -
	D100bA	- - - - - A - - - - - - - - - - - - -
	D100bE	- - - - - E - - - - - - - - - - - - -
	S100cA	- - - - - A - - - - - - - - - - - - -
	P100dA	- - - - - A - - - - - - - - - - - - -
	Q100eA	- - - - - A - - - - - - - - - - - - -
	Q100eN	- - - - - N - - - - - - - - - - - - -
	Q100eF	- - - - - F - - - - - - - - - - - - -
	D100fA	- - - - - A - - - - - - - - - - - - -
	D100fE	- - - - - E - - - - - - - - - - - - -
	N100gA	- - - - - A - - - - - - - - - - - - -
	N100gD	- - - - - D - - - - - - - - - - - - -
	N100gQ	- - - - - Q - - - - - - - - - - - - -
	N100gH	- - - - - H - - - - - - - - - - - - -
	N100gY	- - - - - Y - - - - - - - - - - - - -
	Y100hA	- - - - - A - - - - - - - - - - - - -
	Y100hF	- - - - - F - - - - - - - - - - - - -
	Y100hW	- - - - - W - - - - - - - - - - - - -
	Y100iA	- - - - - A - - - - - - - - - - - - -
	Y100iF	- - - - - F - - - - - - - - - - - - -
	Y100iW	- - - - - W - - - - - - - - - - - - -
	M100jA	- - - - - A - - - - - - - - - - - - -
	D101A	- - - - - A - - - - - - - - - - - - -
	V102A	- - - - - A - - - - - - - - - - - - -
	3D3A	- - - - - A A - - - - A - - - - - - -
	3D2N	- - - - - N N - - - - A - - - - - - -
	b7*	- - - - - T - - - - - - - - - - - - -
	MV2*	- - E - K - - N - - - - - - - - - -
	3B1**	- - Q W N - - - - - - - - - - - - -
	3B2**	- - W T - - - - - - - - - - - - -
	3B3**	- - E W G - - - - - - - - - - - - -
	3B4**	- - W N - - - - - - - - - - - - -
	3B6**	- - L W N - - - - - - - - - - - - -
	3B7**	- - S W R - - - - - - - - - - - - -
	3B9**	- - W R - - - - - - - - - - - - -
	H1.3B/H3.32**	- - E W G - E Q F R F - - - - - - -
	H1.3B/H3.33**	- - E W G - E M F R Y - - - - - - -
	H1.3B/H3.34**	- - E W G - E M R R F - - - - - - -
	H1.3B/H3.35**	- - E W G - H Q R R Y - - - - - - -
	H1.3B/H3.36**	- - E W G - Q R R Y - - - - - - -
	H1.3B/H3.38**	- - E W G - T Q R R F - - - - - - -
	H1.3B/H3.39**	- - E W G - Q V R Y - - - - - - -
	CS**	- - E W T - - - - - - - - - - - - -
	CS**	- - E W T - - F - - - - - - - - - -
	CS**	- - E W T - - M D - - A - - - - - -

B	Cl n ID	CDR H1
	b12 (w.t.)	G Y R F S N F V
	Solvent access#	2 0 3 0 2 1 1 0
	3B3#	- - - - - - - - T
	H31L42#	- - - - - H - T
	"germline"	- - T - T S Y A

C	Clone ID	CDR H2
	b12 (w.t.)	I N P Y N G N K
	Solvent access#	0 1 0 3 2 3 3 2
	N52A	- A - - - - -
	P52aA	- - A - - - -
	Y53G	- - - G - - -
	N56A	- - - - - A -
	"germline"	- - A G - - - T

D	Clone ID	CDR L1
	b12 (w.t.)	H S I R S R R
	Solvent access#	2 2 0 3 2 2 1
	R29S	- - - S - - -
	S30A	- - - - A - -
	R31S	- - - - - S -
	R32Y	- - - - - Y
	b21*	- N - - - - -
	H31L42**	- Q L D G S -
	"germline"	Q - V S - S Y

E	Clone ID	CDR L2
	b12 (w.t.)	G V S
	Solvent access#	1 1 2
	"germline"	- A -

F	Clone ID	CDR L3
	b12 (w.t.)	Q V Y G A S S Y T
	Solvent access#	0 0 0 0 3 3 1 0 1
	A93Y	- - - - Y - - - -
	S94A	- - - - A - - - -
	b7*	- Q - - S - R - -
	H31L42**	- Q - - W P F - -

FIG. 1. Amino acid sequences of mutants and variants of wild-type (w.t.) b12 in CDR loops H3 (A), H1 (B), H2 (C), L1 (D), L2 (E), and L3 (F). Each panel shows the solvent accessibility (Solvent access#) of the side chains of each residue, the relevant sequences of mutants of b12 engineered for this study, and natural (*) and in vitro-evolved (**) variants isolated in previous studies (references 4 and 57 and references 5 and 90, respectively). The solvent accessibilities of the side chains were determined by the NACCESS computer program (16) and are graded as follows: 0, buried (<10% exposure); 1, partially exposed (10 to 35% exposure); 2, moderately exposed (36 to 66% exposure); 3, mostly exposed (67 to 90% exposure); and 4, fully exposed (>90% exposure). H31L42 (37) is a whole-IgG version of the Fab designated h1.1 h3.33/L1.4L3.14 (90). The deduced amino acid sequences of the closest related germ line DNA are shown for comparison; the germ line sequences for the heavy and light chains are IGHV1-3*01 (DP-25; accession no. X62109) and IGKV3-20*01 (DPK22; accession no. X12686), respectively.

son) diluted 1:1,000 in PBS containing 1% BSA and 0.025% Tween 20 was added. After a 30-min incubation at room temperature, the plates were washed, and the tetramethylbenzidine (TMB) substrate kit (Pierce) was used for developing, according to the manufacturer's instructions. The optical density at 450 nm was read on a microplate reader (Molecular Devices), and the results were recorded as the 50% inhibitory concentration (IC_{50}), defined as the concentration of competing Fab that was required to reduce the maximal signal generated by biotinylated Fab b12 by 50%. All competition ELISAs were performed twice.

Synthesis of b12 CDR H3 peptide and coupling to BSA. A peptide corresponding to CDR H3 of b12 was synthesized with the b12 crystal structure as a guide (66); the peptide has the sequence C-PGK-A⁹³RVGPYSWDDSPQD NYYMDW¹⁰³. Residues PGK were included to promote a type II β -turn (31), and a Cys residue was added to facilitate cyclization via native chemical ligation (19) and chemical coupling to a carrier molecule. The Arg 94 side chain in the third framework region (FR3) projects into solvent in the crystal structure and so was included in the peptide, as was Ala 93. The peptide was synthesized with *N*-tert-butoxy-carbonyl chemistry (71), purified by reverse-phase high-pressure liquid chromatography to >95% purity, and verified by mass spectrometry.

To cyclize the peptide, the backbone was linked N to C terminus by native chemical ligation (28), and the final product was purified by reverse-phase high-pressure liquid chromatography to >95% purity and verified by mass spectrometry. The free Cys of the peptide was used to cross-link the peptide to activated BSA (Pierce) by using the manufacturer's instructions. Briefly, 10 mg of sulfo-succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate-activated BSA in 1 ml of deionized H₂O was mixed with 10 mg of peptide dissolved in 0.25 ml of dimethyl sulfoxide and 1.75 ml of PBS. The mixture was rocked gently for 2 h at room temperature, dialyzed extensively against PBS, and then sterilized with a 0.2- μ m filter. A control peptide (GTP-binding peptide of sequence CEGN-VRSRELAGHTGY; American Peptide Co.) was similarly linked to BSA. The protein concentration of each sample was determined with the Micro BCA protein assay kit (Pierce) according to the manufacturer's instructions. The conjugates were also analyzed by SDS-PAGE, and cross-linking was confirmed by an increase in the molecular weight of the activated BSA after reacting with the peptide; both the b12 CDR H3 and the control peptide caused a similar shift in molecular weight.

HIV-1 neutralization assay. The primary isolate HIV-1_{JRFL} was assayed for neutralization with peripheral blood mononuclear cells as target cells and detection of p24 in ELISA as a reporter assay, as described previously (94).

Nucleotide sequence accession number. The sequences of the heavy and light chains of b12 have been deposited in GenBank (accession no. AAB26315.1 and AAB26306.1, respectively).

RESULTS

Alanine-scanning mutagenesis of CDR H3 of b12. In the mutagenesis strategy, we chose to compare binding of different mutants of b12 to gp120 with crude Fab supernatants prepared from bacterial cultures (3), which facilitated the rapid analysis of a large number of Fab mutants (Fig. 1) that otherwise would not have been feasible. Apparent affinities relative to wild-type Fab b12 were measured by ELISA, as described in Materials and Methods. As a first step in our analysis of b12, we performed a complete alanine scan of the CDR H3 loop and tested the mutant Fabs by ELISA to determine their relative binding strengths to gp120. We chose recombinant gp120 from two different strains, one from a primary isolate (gp120_{JRFL}) and one from a T-cell-line-adapted strain (gp120_{IIIB}), to see whether any differences in binding could be found with our mutant Fab panel. We also included in our analysis the previously described, b12-specific peptide B2.1, as displayed on filamentous phage (93). The dissociation constant (K_d) of wild-type Fab b12 against gp120_{LA1} is ≈ 9 nM (62), against the B2.1 synthetic peptide is ≈ 2.5 μ M (93), and against B2.1 phage is closer to the K_d of the b12-gp120 interaction because of the constraint that the phage coat imparts to the peptide, although

a precise K_d value of b12 for the phage-associated peptide is difficult to determine (93).

The results of the alanine scan of the H3 loop of b12 are shown in Fig. 2. Substitution of 7 out of 18 residues in H3 with Ala reduced binding of Fab b12 to both gp120_{JRFL} and gp120_{IIIB} by greater than 90%. The relevant mutants are V95A, Y98A, W100A, Q100eA, N100gA, Y100hA, and Y100iA. [The CDR H3 of b12 contains a 10-residue insertion. In Kabat and Wu numbering (34), these residues are designated 100A, 100B, . . . , 100J. For clarity, these inserted residues will be denoted with a lowercase letter after the number of the residue position so as to avoid confusion with the mutated residue (uppercase) when referring to mutations (e.g., mutation D100aA)]. The loss of antigen binding of the W100A mutant was not unexpected from the crystal structure of IgG1 b12 and a docking model of b12 and the gp120 core (66). However, the behavior of some of the other mutants was more surprising.

Substitutions in b12 that reduced gp120 binding as much as or even more than observed with the W100A mutant included V95A, Y98A, Q100eA, N100gA, Y100hA, and Y100iA. Most of these substitutions are at the base of the CDR H3 and involve side chains that are poorly exposed to solvent, as defined in Fig. 1. Solvent accessibility to side chain (Fig. 1) was not, however, predictive of the effect of a substitution on antigen binding (Fig. 2). The greatest effects of an Ala substitution on gp120 recognition occurred with residues N100g, Y100h, and Y100i at the C-terminal end of H3, which resulted in complete loss of gp120 recognition in our assay (<0.1% apparent affinity relative to wild-type b12). We confirmed these results by purifying Fab protein for these mutants and were unable to observe significant reactivity with gp120 even at 20 μ g/ml (data not shown). To further explore these findings, additional substitutions were made in these positions and tested for binding to gp120 and the B2.1 peptide (see below). Significantly, there was generally good agreement in the relative binding of Ala mutants to gp120_{JRFL} (primary isolate) and gp120_{IIIB} (T-cell-line-adapted).

Thirteen Ala substitutions in H3 had qualitatively similar effects on the ability of b12 to bind to gp120 and B2.1, suggesting some similarity in recognition of these antigens by b12. However, the effects of other Fab substitutions such as Y98A, W100A, D100aA, and D100bA did not correlate, pointing to possible ways of improving the B2.1 peptide as a b12 epitope mimic.

Mutations in CDR H3 W100. A striking feature in the crystal structure of IgG1 b12 is the prominent display of the aromatic residue W100 at the apex of the long H3 loop (66). As shown in Fig. 2 and 3A, Ala substitution at W100 significantly diminished binding of Fab b12 to gp120_{JRFL} and gp120_{IIIB}. This result was confirmed with purified Fab: the W100A mutant inhibited biotinylated Fab-b12 with an IC_{50} of 29 μ g/ml and 50 μ g/ml for gp120_{JRFL} and gp120_{IIIB}, respectively, compared to an IC_{50} of 3 μ g/ml and 1.5 μ g/ml, respectively, for wild-type Fab b12 (data not shown).

In the b12-gp120 docking model, W100 was acting to fill the hydrophobic cavity of the CD4bs on gp120 in a way analogous to F43 of CD4. Thus, we also made the W100F mutant to determine whether Phe would be more or less favored than Trp at this position. Retention of an aromatic ring in the

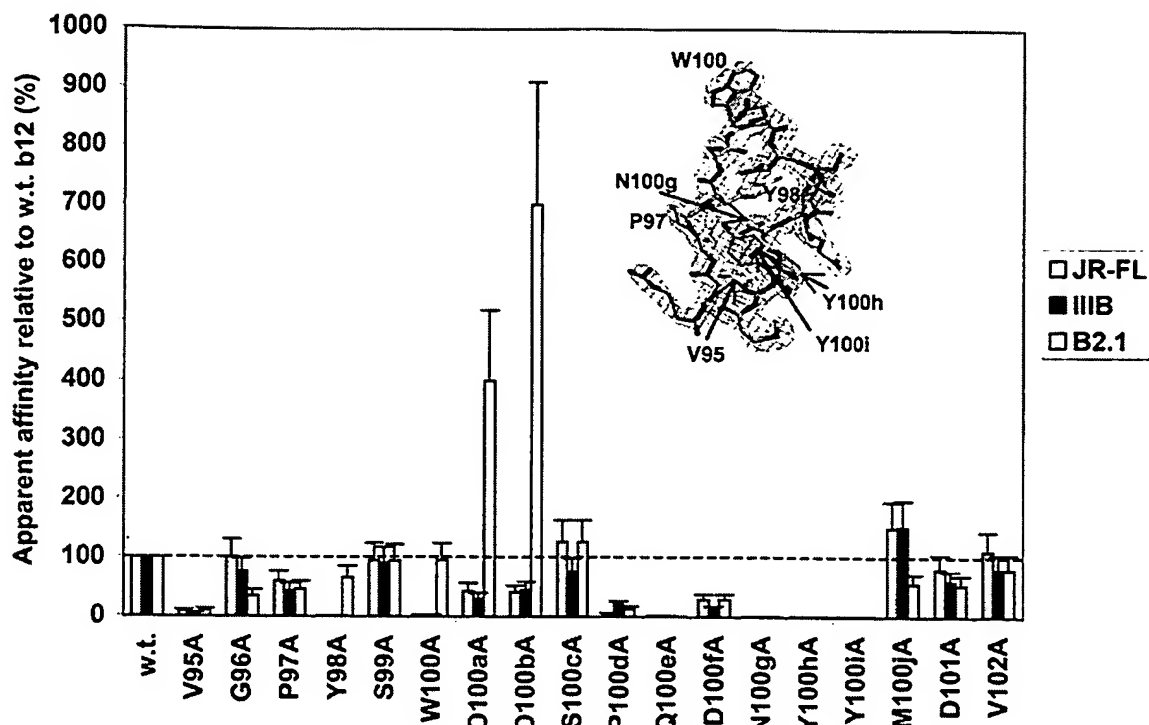


FIG. 2. Alanine-scanning mutagenesis of CDR H3 of b12. Bars indicate the apparent affinities of Fab mutants relative to wild-type (w.t.) Fab b12 for gp120_{JR-FL}, gp120_{IIIB}, and the B2.1 peptide. The H3 loop and corresponding electron density from the intact b12 structure (66) are shown (inset), with key residues indicated.

W100F mutant did promote slightly better binding than the W100A mutant to both gp120s, but the apparent affinity was $\approx 10\%$ relative to that of the wild type. Two other mutants, W100S and W100V, also had considerably impaired binding to both gp120s. Taken together, these results confirm the importance of W100 for b12 by showing that Trp is preferred over four other residues, including another aromatic, Phe, at this position. In contrast to the results with the gp120s, the W100 mutants were all able to bind B2.1 at nearly wild-type levels, strongly suggesting that W100 is not involved in B2.1 recognition.

Role of Asp residues in CDR H3 of b12. Another feature of the b12 crystal structure that initially drew our attention was a clustered grouping of acidic moieties on one face of the H3 loop (66). We wondered whether this "acidic patch" was involved in keeping the H3 loop of b12 erect via charge repulsion with a weakly acidic patch near the base of the H3 loop. The residues involved included D100a, D100b, and D100f, as well as hydroxyl groups from Ser 99, Ser 100c, and Tyr 100i (D101 was not involved in this patch and moreover was found to have little involvement in binding to gp120 and B2.1; see Fig. 2). The Ala substitutions (Fig. 2) had mostly moderate effects on binding to gp120 (≈ 2 - to 3-fold-reduced binding relative to wild-type b12). Further mutagenesis (Fig. 3B) yielded mutants D100aE, D100bE, and D100fE, the substitutions in which either had no effect (D100aE) or again only moderate effects (D100bE and D100fE) on binding to gp120. Two additional mutants were constructed in which all three positions were changed to see if there was any cooperativity among these

residues. A triple Ala mutant, dubbed 3D3A, and another triple mutant, D100aN/D100bN/D100fA, dubbed 3D2N, were still able to consistently bind to gp120_{JR-FL} and gp120_{IIIB}, albeit at somewhat lower levels (data not shown).

The acidic patch on the paratope of b12 appears to have significance for B2.1 recognition. The Ala mutants D100aA, D100bA, and both triple mutants bound to B2.1 much better than to wild-type Fab b12. Interestingly, the D100fE mutant bound extremely poorly to B2.1 but bound gp120 almost as well as the wild type. In fact, the D100fE mutant bound more poorly to B2.1 than the "less conservative" D100fA mutant, revealing a very distinct requirement for B2.1 recognition at position 100f.

Mutagenesis in CDR H3 of b12 N-terminal to W100. Four residues N-terminal to W100 in H3 of b12 were chosen for additional study. V95 was chosen because the Ala mutation at this position caused severe impairment in binding to gp120, and we wished to determine whether a more conservative substitution with Ile would also reduce binding. Figure 3C shows that the V95I mutant bound gp120 at nearly wild-type levels. The extra bulk of a branched aliphatic side chain at this position is probably necessary for full activity of b12. The residues P97, Y98, and S99 were also targeted for further mutagenesis, inspired by previously published variants of b12 (both naturally occurring and in vitro-enhanced variants), including 3B3, in which the sequence EWG is found in place of PYS at these positions (4, 5) (Fig. 1A). The Y98W mutant indeed bound ≈ 3 - to 9-fold better to the gp120s than did wild-type Fab b12 (Fig. 3C). The P97E and S99G mutants

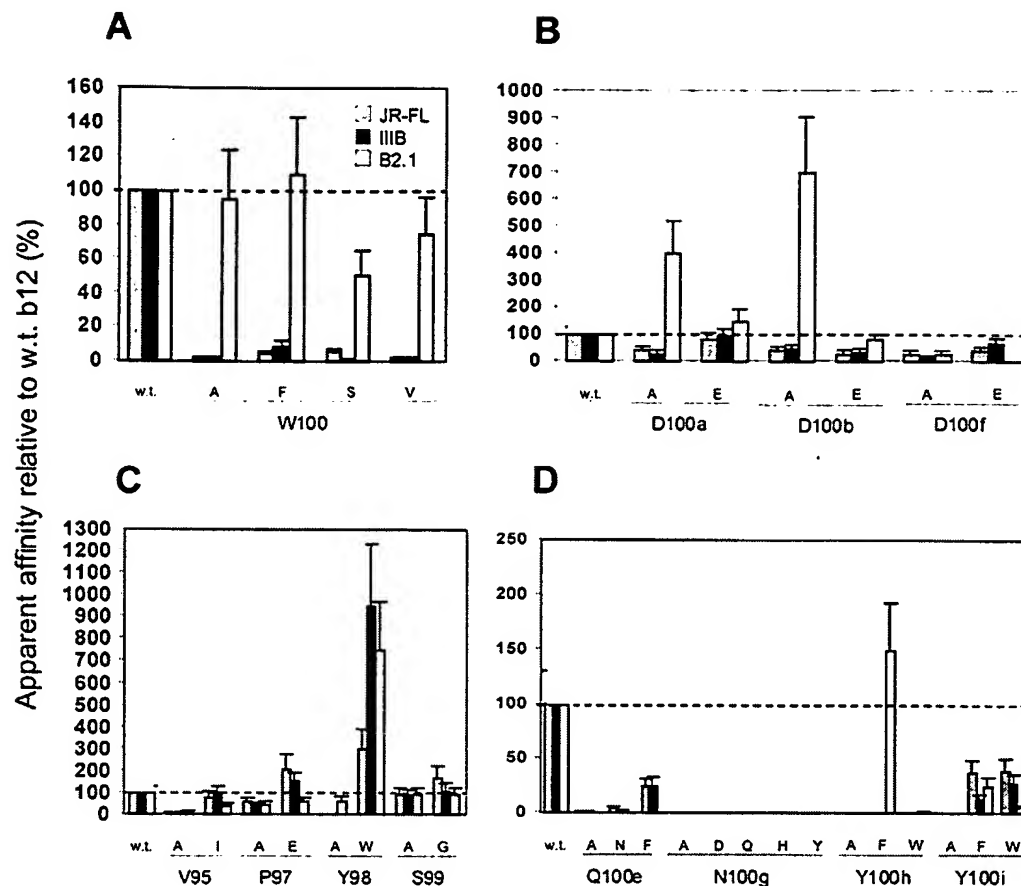


FIG. 3. Further substitution analysis of residues in the H3 loop of b12. Bars indicate the apparent affinities of Fab mutants relative to wild-type (w.t.) Fab b12 for gp120_{JRFL}, gp120_{IIIB}, and the B2.1 peptide. Substitutions were made to Trp100 (A), Asp residues near the crown of the H3 loop (B), selected H3 residues N-terminal to Trp100 (C), and key residues C-terminal to Trp100 (D).

bound to gp120 as well as or only slightly better than wild-type b12. Thus, the enhanced binding of 3B3 to gp120 presumably derives mostly from the preference for a W over a Y at the middle position of the EWG motif, with the flanking residues perhaps providing additional fine tuning.

In contrast to the Ala mutant, P97A, B2.1 binding was not correlated with gp120 binding for mutant P97E. Interestingly, the substitution Y98W enhanced the binding of Fab b12 to both B2.1 and gp120, whereas the S99G substitution was silent for both gp120 and B2.1.

Mutagenesis in CDR H3 of b12 C-terminal to W100. The alanine scan (Fig. 2) showed that residues Q100e, N100g, Y100h, and Y100i in the C-terminal portion of H3 of b12 were all important for gp120 recognition and were therefore chosen for further mutational analysis. Q100eN and Q100eF mutants also bound gp120 with diminished affinity relative to wild-type Fab b12, although the gp120 binding was significantly improved for Q100eF relative to Q100eA (Fig. 3D). For verification purposes, Fab Q100eA was purified and used in a competition ELISA with biotinylated Fab b12 against gp120_{IIIB}; the IC_{50} of Q100eA was ≈ 14 μ g/ml, which is nine times greater than that of wild-type Fab b12 ($IC_{50} \approx 1.5$ μ g/ml) and consistent with the crude Fab ELISA. In the structure of IgG1 b12, the side chain of Q100e is only partially accessible to solvent

and makes a hydrogen bond with the main chain of A93. A loss of this hydrogen bond might destabilize the interaction at the base of CDR H3 and could explain the observed reduction in binding to gp120 for the Q100e mutants.

Next, we tested more conservative substitutions at position N100g. Neither an N100gD nor an N100gQ mutant was able to bind either gp120_{JRFL} or gp120_{IIIB}, indicating that the removal of an amino group or the addition of a methylene group, respectively, from the side chain of Asn100g was sufficient to completely abolish gp120 recognition in our assay format (Fig. 3D). In the crystal structure of b12, the amino nitrogen on the side chain of Asn100g makes a hydrogen bond with the main-chain carbonyl of Gly96; indeed, the side chain of Asn100g is $<10\%$ exposed to solvent (Fig. 1). Again, mutations in Asn100g most likely affect the structure of the paratope of b12 due to the absence of the stabilizing hydrogen bond. Thus, not surprisingly, the substitution of Asn100g with either His or Tyr completely abolished binding to either gp120_{JRFL} or gp120_{IIIB}. The Tyr substitution was chosen because the JH6 family of J segments (the J-segment family used by b12 in VDJ-recombination) encodes a repeating string of Tyr residues, and thus b12-like antibodies that also use the JH6 family might encode a Tyr at this position. The complete lack of detectable binding to gp120 by any of the 100g mutants underscores the critical

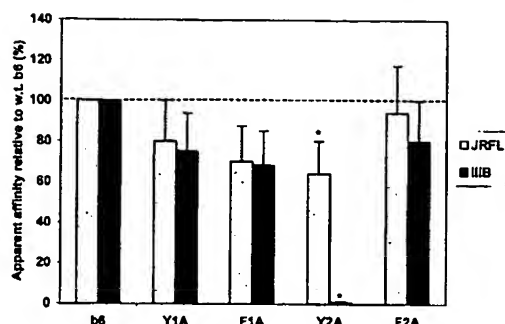


FIG. 4. Effect of alanine substitutions of the four aromatic residues in the H3 loop of b6 on relative binding of Fab b6 to gp120_{JR-FL} and gp120_{IIIB}. Bars indicate the apparent affinities of Fab mutants relative to wild-type (w.t.) Fab b6 for gp120_{JR-FL} and gp120_{IIIB}. The amino acid sequence of the H3 loop of b6 is OKPR¹Y¹F¹DLLSGQY²RRVAG AF²DV (the aromatic residues are in boldface and have superscript numbers to correspond with the bar graph). By ELISA, the half-maximal binding of the purified Fab of the Y²A mutant to gp120_{IIIB} was 0.4 μ g/ml, which was \approx 50 times lower than the half-maximal binding of mutant Fab Y²A to gp120_{JR-FL}, 0.007 μ g/ml (*); half-maximal binding of wild-type Fab b6 to both gp120_{IIIB} and gp120_{JR-FL} was determined to be 0.003 μ g/ml (data not shown).

importance of residue N100g in the activity of b12 (see the Discussion).

In contrast to the irreplaceability of N100g, antigen recognition was found to be at least partially maintained with conservative substitutions to Y100h and Y100i. Thus, the Y100i(F/W) mutants bound to gp120 at \approx 20% to 40% wild-type levels (Fig. 3D). These results imply that a bulky aromatic at position 100i is required by Fab b12 for recognition of gp120 but that the added hydroxyl group of Tyr100h is important for full antigen binding. By contrast, only a Tyr residue appears to be sufficient for gp120 recognition at position 100h, but the Y100hF mutant was able to bind B2.1 at wild-type levels.

Aside from mutant Y100hF, all the other b12 H3 mutants (conservative mutations C-terminal to W100) had impaired binding to B2.1. Interestingly, a Phe substitution at Y100h but not at Y100i fully restored B2.1 binding. This may reflect the observation that the two Tyr residues are pointing in roughly opposite directions in the crystal structure of b12.

Substitution of aromatic residues in H3 loop of nonneutralizing anti-CD4bs antibody b6. Following our analysis of the H3 loop of b12, we were particularly interested in the role of aromatics because substitution to Ala of all four aromatics in the H3 loop of b12, not only W100, caused a >95% decrease in relative binding to gp120. Thus, we chose to see if H3 loop aromatics were important in the function of another anti-CD4bs antibody, b6, which, like b12, has a long H3 loop with four aromatic residues but does not neutralize primary isolates of HIV-1 (62). The sequence of the H3 loop of b6 is given in Fig. 4, and the aromatic residues are designated Y¹A, F¹A, Y²A, and F²A. The results (Fig. 4) indicated that in contrast to b12, mutation to Ala of only one of the four aromatics in the H3 of b6, Y²A, led to a severe decrease in binding of Fab b6 to gp120 and only to gp120_{IIIB} and not gp120_{JR-FL}. In fact, the Y²A substitution decreased the half-maximal binding of purified Fab b6 to gp120_{IIIB} from 0.003 μ g/ml to 0.4 μ g/ml, which was far more severe than the change in half-maximal binding to

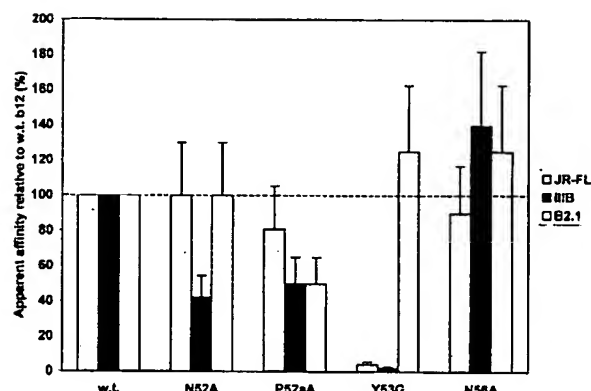


FIG. 5. Effect of substitutions in the H2 loop of b12 on relative binding of Fab b12 to gp120_{JR-FL}, gp120_{IIIB}, and the B2.1 peptide. Bars indicate the apparent affinities of Fab mutants relative to wild-type (w.t.) Fab b12 against gp120_{JR-FL}, gp120_{IIIB}, and the B2.1 peptide. The mutations P52A and Y53G were back mutations to the residues encoded by the closest related germ line DNA (see Fig. 1).

gp120_{JR-FL} (from 0.003 μ g/ml to 0.007 μ g/ml; data not shown). The differences in the roles of H3 aromatics between b12 and the poorly neutralizing antibody b6 are striking and are discussed below.

b12 H2 mutations. In the b12-gp120 docking model (66), it was predicted that residues in H2 could contact gp120. Residue Y53 is the most prominent in H2, points directly toward gp120 in the model, and occupies a large space in the b12-gp120 interface. We suspected that residue P52a might also play a role in gp120 recognition by maintaining the H2 loop in a particular conformation rather than by making extensive contact with gp120. Interest in residues P52a and Y53 was also strong because these were nonconservatively mutated from the residue encoded by the closest germ line genes at these positions, Ala and Gly, respectively. The germ line genes closest to b12 are DPK22 and DP-25 (<http://imgt.cnusc.fr:8104/> [17, 78]) for the light and heavy chains, respectively, and the residues they encode are shown for each CDR in Fig. 1. The strategy of using germ line "back mutations" for evaluating residues outside of H3, where somatic mutation is key to generating residue diversity, was adopted to determine the dependence of b12 binding on somatic mutation. Residues N52 and N56 were also in the interface between b12 and gp120 and thus were targeted for mutagenesis.

With the exception of Y53G, the H2 substitutions had only moderate to slight effects on Fab binding to gp120_{IIIB}, gp120_{JR-FL}, and B2.1 (Fig. 5). Residue N52 was predicted to make relatively minor contact with gp120, and correspondingly the N52A substitution only moderately diminished binding against gp120_{IIIB}. Interestingly, the N56A mutant bound gp120 at wild-type levels (Fig. 5), implying that this residue does not significantly contribute to gp120 binding. Residue N56 docks in close proximity to residue K362 of gp120; however, the side chain of N56 points away from the contact surface, potentially accounting for the absence of an effect on gp120 binding of the N56A substitution. The P52aA substitution was expected to affect H2 loop structure rather than replace a contact residue, and the effect on gp120 binding was moderate (i.e., \approx 2-fold reduction in apparent affinity for gp120), indicating that P52a

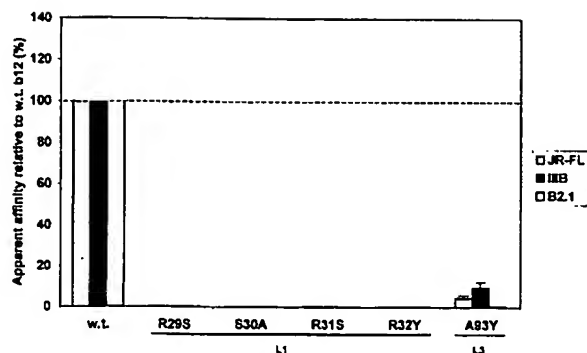


FIG. 6. Effect of substitutions in the CDR loops L1 and L3 of b12 on the relative binding of Fab b12 to gp120_{JR-FL}, gp120_{IIIB}, and the B2.1 peptide. Bars indicate the apparent affinities of Fab mutants relative to wild-type (w.t.) Fab b12 for gp120_{JR-FL}, gp120_{IIIB}, and the B2.1 peptide. The mutations R29S, R31S, and R32Y were back mutations to the residues encoded by the closest related germ line DNA (see Fig. 1).

has a rather modest role in gp120 binding. The Y53G substitution, by contrast, greatly diminished the binding of b12 to gp120, suggesting that Y53 contributes significantly to the binding energy between b12 and gp120. Residue Y53 protrudes to form a second "finger" in the paratope of b12 and was predicted to bury into a canyon in gp120 (see Discussion). The Y53G substitution had little effect on B2.1 recognition, implying that B2.1 does not contact Y53, considering that the side chain of Y53 is mostly solvent exposed (Fig. 1). We note that attempts to purify the Y53G mutant resulted in a somewhat impure Fab preparation that bound poorly to gp120 and B2.1 (data not shown), whereas the crude Fab bound B2.1 at wild-type levels, suggesting that this mutant might be unstable to the purification conditions, which involve acid elution.

b12 L1 and L3 mutations. Finally, the b12-gp120 docking model indicated that various residues in CDRs L1 and L3 could play a role in binding to gp120. Residues R29, R31, and R32 in L1 were chosen for substitution to S, S, and Y, respectively, because the closest germ line genes encode the substituted residues at these positions. S30 was changed simply to Ala because the closest germ line gene already encodes a Ser at this position. The results indicate that all four of these L1 mutants were essentially unable to recognize gp120 (Fig. 6) and suggest that somatic mutations found in this loop are essential to b12 specificity. Two mutations were made in L3 (A93Y and S94A), also based on predictions of their interaction with gp120. The A93Y mutant suffered a significant loss in gp120 binding (Fig. 6). Fab A93Y was purified and used in a competition ELISA with biotinylated Fab b12 against gp120_{IIIB}; the IC₅₀ of A93Y was $\approx 10 \mu\text{g/ml}$, which is six to seven times greater than that of wild-type Fab b12 (IC₅₀ $\approx 1.5 \mu\text{g/ml}$) and consistent with the crude Fab ELISA. Unfortunately, the S94A mutant was found to be poorly produced in crude bacterial supernatants relative to the wild type (>10 -fold reduction; data not shown), precluding a quantitative analysis of binding of the latter Fab mutant. Nevertheless, binding of the S94A Fab mutant to gp120 was detectable despite the low concentration of Fab in the supernatants, and we were able to conclude that the S94A mutation is at least not a complete knockout mutation (data not shown).

Whereas the mutations in L1 completely knocked out the binding of Fab b12 to both B2.1 and gp120 in our assay, the L3 mutation, A93Y, abolished binding to B2.1, but binding to gp120 was maintained, albeit at $\approx 10\%$ of wild-type levels (Fig. 7). It is unclear whether the residues in L1 contact gp120 or if their mutation disrupts the b12 paratope. However, it appears that A93Y is important for B2.1 recognition because the paratope of the A93Y mutant was sufficiently intact to bind to gp120 with measurable affinity (Fig. 7). As for the poorly produced Fab mutant S94A, the result was similar for B2.1 as for gp120; at most, only a moderate effect on B2.1 binding was expected for this mutation (data not shown), although a quantitative analysis was not attempted.

Neutralization of HIV-1 by synthetic CDR H3 b12 peptide.

We recently reported the neutralization of the T-cell-line-adapted strains HIV-1_{MN} and HIV-1_{IIIB} by a conjugate of BSA and a synthetic peptide corresponding to the H3 loop of b12 (66). This neutralizing activity against HIV-1_{MN} and HIV-1_{IIIB} was specific because a conjugate of BSA and an irrelevant peptide did not neutralize virus (66). We present here some additional observations. Whereas neutralization of HIV-1 was found for HIV-1_{MN} and HIV-1_{IIIB} in H9 cells at $\leq 1 \text{ mg}$ of conjugate per ml (IC₇₅ $\approx 0.5 \text{ mg/ml}$ for each [66]), no neutralization at 4 mg of the peptide-BSA conjugate per ml was observed for the primary isolate HIV-1_{JRFL} in a peripheral blood mononuclear cell assay (data not shown). It should be noted that, in our hands, neutralization (IC₉₀) of HIV-1_{JRFL} occurs at $\approx 0.8 \mu\text{g}$ of IgG1 b12 per ml (94), so HIV-1_{JRFL} is as sensitive to neutralization by IgG1 b12 as HIV-1_{IIIB} (IC₉₀ $\approx 0.5 \mu\text{g/ml}$). Thus, although T-cell-line-adapted viruses were neutralized at high concentrations of the b12 H3 peptide conjugate, the primary isolate, HIV-1_{JRFL} was not neutralized by the same conjugate.

We also wished to determine whether a direct interaction between the b12 H3 peptide and recombinant gp120 could be established. By direct ELISA, no specific interaction between the b12 H3 peptide or the b12 H3 peptide-BSA conjugate and gp120 was detected by immobilizing either the peptide or recombinant gp120 (JRFL and IIIB) and then probing with the partnering molecule (data not shown). Similarly, by competition ELISA, high concentrations of b12 H3 peptide (0.5 mg/ml) or b12 H3 peptide-BSA conjugate (4 mg/ml) did not inhibit the ELISA signal generated by Fab b12 against immobilized recombinant gp120 (JRFL and IIIB; data not shown). Thus, it appears that, although the b12 H3 peptide-BSA conjugate neutralizes the T-cell-line-adapted viruses HIV-1_{MN} and HIV-1_{IIIB}, we could not demonstrate a specific interaction between the conjugate and recombinant gp120, at least by direct and competition ELISAs, which may not detect interactions with high micromolar to millimolar dissociation constants. These results may be explained at least in part by differences in the conformation of gp120 as it exists in the trimeric envelope spike, as probed in neutralization assays versus recombinant, plate-immobilized gp120, as probed by ELISA.

DISCUSSION

In the current study, we examined the antigen binding site of b12 with site-directed mutagenesis to create 50 mutations involving 27 different residues in four different CDRs of b12. We

found that the use of crude Fab supernatants was reproducible and efficient, allowing the simultaneous analysis of a large number of Fab mutants against a panel of antigens. This type of mutational analysis can easily be adapted for other Fabs or single-chain Fvs that are amenable to production in *E. coli*, and we are currently using this approach for other antibodies against HIV-1. One potential drawback of the analysis is that if a substitution leads to diminished antigen binding, it is not known whether it is due to a direct effect on affinity for antigen or an effect on Fab stability or folding. However, we targeted residues in the CDR loops, most of which were at least partially exposed to solvent. For buried residues such as V95, N100g, and Y100i, it is possible that Ala substitution might partially unfold these mutants. Nevertheless, of the mutants that were chosen to be purified, W100A, Q100eA, N100gA, Y100hA, A93Y, and Y53G, most gave favorable yields of Fab and produced a single 50-kDa band by SDS-PAGE, suggesting that the Fabs were largely intact and not degraded. Moreover, the purified Fabs generally showed activities against gp120 very similar to those of the crude Fabs.

A very strong correlation was found for binding of the mutants to both gp120_{IIB} and gp120_{JR-FL}. This correlation is consistent with a common binding mechanism of b12 to both the T-cell-line-adapted and primary isolate gp120 and is perhaps not surprising for an antibody with such broad reactivity to diverse HIV-1 envelopes (11, 54, 79). W100 was found to be important, as its substitution generally decreased binding of b12 to gp120. This result is also consistent with a prior experiment in which a portion of the gene segment encoding the H3 loop of b12, including W100, was randomized and incorporated into a phage display library and the library was affinity selected against gp120. A Trp residue at position 100 was absolutely conserved in all clones selected (5). These data caused us to speculate that perhaps a Trp residue (b12) might be superior to a Phe (CD4) in filling the hydrophobic pocket in gp120 that is important for CD4 receptor engagement (Phe43 is used to fulfill this role in CD4 [39]). However, an F43W mutant of CD4 did not show enhanced binding to gp120 but rather bound more poorly (i.e., the F43W mutant bound to gp120 at $\approx 9\%$ of wild-type CD4 levels, and F43Y bound gp120 at $\approx 40\%$ of wild-type CD4 levels; Raymond Sweet, personal communication). In addition to W100, the three remaining aromatics in the H3 loop of b12 appeared to be important for gp120 recognition; mutation of any of four aromatics (Y98, W100, Y100h, and Y100i) to Ala resulted in $\geq 95\%$ decrease in relative binding to gp120.

By contrast, mutation to Ala of only one of four aromatics (i.e., residue Y², as defined in Fig. 4) in the H3 loop of the poorly neutralizing anti-CD4bs antibody b6 resulted in a $\geq 95\%$ decrease in relative binding to gp120 and then only to one of the two strains, IIB (Fig. 4). In fact, for the Y²A mutant of Fab b6, a much greater binding differential was found between gp120 strains than for any of the 50 b12 mutants (Fig. 4). This result highlights a striking difference in the way that the aromatics of b12 and b6 are used to bind gp120, at least with respect to their H3 loops, even though the footprints of b6 and b12 on gp120 appear to be relatively similar (55). The H3 loop of an antibody is usually crucial in determining its specificity (82, 89). It may be that W100 (b12) and Y² (b6), each of which is located near the middle of their long H3 loops, probe dif-

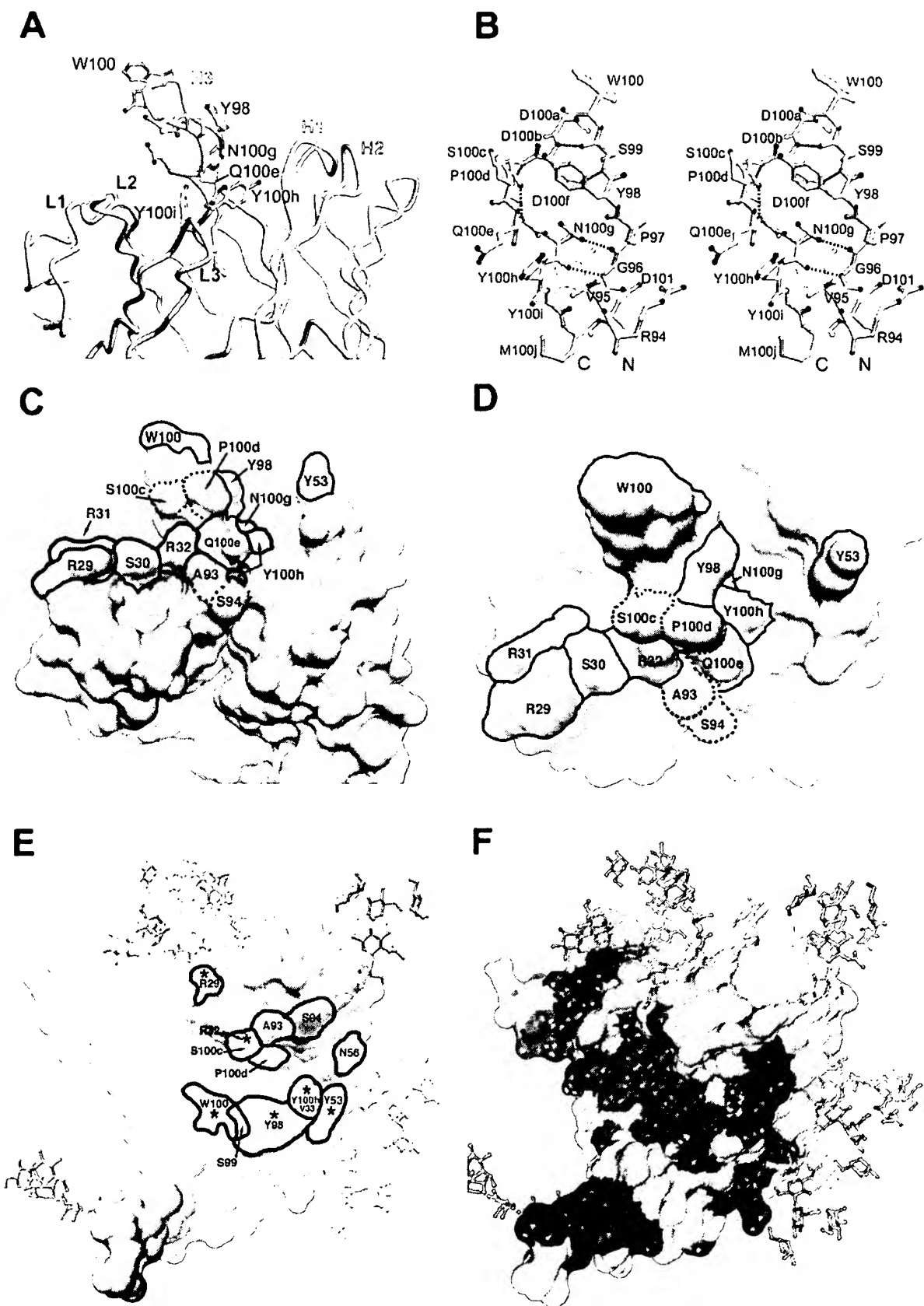
ferent regions within the CD4bs of gp120. Broadly neutralizing molecules such as b12 and CD4 might be able to occupy the conserved hydrophobic pocket of gp120, whereas b6 may not possess this ability. We speculate that the b6 H3 loop lacks rigidity and might lie across the CD4bs of gp120 rather than inserting into it like b12. Given that the Y²A mutation of b6 leads to significant strain preference (Fig. 4) but W100A of b12 does not, the Y² residue could be interacting with or proximal to a region of gp120 that is relatively variable.

Recently, Zhu et al. showed that an engineered molecule (MBri) containing the V1/V2 loop and a portion of the bridging sheet of gp120 (i.e., $\beta 2$, $\beta 3$, $\beta 20$, and $\beta 21$) was able to bind to b12 (91). These authors showed that a V3 loop peptide is able to partially inhibit the binding of b12 to MBri and attributed this effect to a physical interaction between the V1/V2 loop and V3 loop. One might speculate that b12 is able to bind to gp120 in spite of an interaction between variable loops on the native trimer, whereas other anti-CD4bs antibodies, such as b6, cannot. In this vein, we have observed that most anti-CD4bs antibodies but not b12 inhibit the binding of a novel loop-dependent antibody to gp120 (MBZ; Robert Kelleher, Richard Jensen, Aran Labrijn, Meng Wang, Gerald Quinnan, Paul W. H. I. Parren, and Dennis R. Burton, submitted for publication), suggesting that the variable loops affect other anti-CD4bs antibodies in a manner different from how they affect b12.

Further comparative studies, both structural and functional, between b12 and other nonneutralizing anti-CD4bs antibodies like b6 could help elucidate the conformational differences between monomeric gp120 and trimeric gp120 on the HIV-1 envelope spike. In terms of vaccine design, these types of analyses should be extremely helpful to the design of improved gp120 constructs that would elicit b12-like antibodies by maximizing the exposure and immunogenicity of the b12 epitope while limiting the antibody response against overlapping epitopes targeted by b6 and other poorly neutralizing anti-CD4bs antibodies.

A molecular (ribbon) model of the Fab of b12 (Fig. 7A) illustrates the relative orientations of the CDRs and the prominent H3 loop. Figure 7B details the contour of the H3 loop and how the H3 polypeptide extends down on either side from W100 in an extended β -ladder with a distinct twist. The β -ladder is roughly 4 Å in width and contains five hydrogen bonds between strands; most notably, the main-chain carbonyl of G96 hydrogen bonds with the amino group of the side chain of N100g. Our data strongly suggest that this stabilizing hydrogen bond is crucial for the interaction of b12 with both gp120 and B2.1, since mutation of N100g to A, D, Q, H, or Y completely abolishes b12 binding to gp120 and B2.1. Another potentially loop-stabilizing hydrogen bond exists between Q100e and A93, and we found that changing Q100e to Ala, Asn, or Phe resulted in diminished binding to gp120. We note that in a recent and related study by McHugh et al. (47), it was shown by mutational analysis of a single chain Fv fragment corresponding to a b12 variant, 3B3-PE, that gp120 recognition was enhanced ≈ 2 -fold by a Q100eY mutation. Thus, it appears that an aromatic residue at Q100e is compatible with strong gp120 binding in certain contexts.

The last few C-terminal residues of the H3 loop of b12 are encoded by DNA contributed by the joining region, which, in



the case of b12, belongs to the family JH6 (4). The JH6 segment of DNA can potentially contribute up to six consecutive Tyr residues. We considered the possibility that this portion of the H3 loop of b12 may be a part of a more conserved and general structural motif, since a single segment of DNA encodes it as a group. We performed a database search (1) on the motif NYMDV and found only three exact matches: one is an antihapten human heavy chain, VH-37 (accession no. S46393 [25]), and two are highly homologous antibodies against rhesus D blood group antigen (accession no. Y08177 and Y08186; S. M. Miescher, personal communication). Unfortunately, no structure for these antibodies is currently available to compare with that of IgG1 b12.

The strong dependence of b12 on a CDR of the light chain is consistent with a previous chain-shuffling experiment in which the heavy chain of b12 exclusively paired with the same light chain following affinity selection on gp120 (4). Significantly, of the six non-H3 mutations that were found to decrease gp120 binding by $\geq 90\%$, four arose via somatic mutation, and three of these were in L1. [The four substitutions, G53Y(H2), S29R, S31R, and Y32R, (L1) were determined to have arisen via somatic mutation on the basis of sequence alignment (<http://imgt.cnusc.fr:8104/home.html>) with known human germ line genes (see Fig. 1), and their positions in the paratope of b12 can be seen in Fig. 7C and D.] Although germ line antibodies can neutralize some viruses (35), given our results, it is very unlikely that a nonsomatically mutated version of b12 would have any HIV-1-neutralizing activity.

In a prior experiment, the gene segment encoding six residues in L1 (including R29, R31, and R32) was randomized and incorporated into a phage display library, and the library was affinity selected against gp120. Although there was positive selection for Arg residues in the enriched phage pools, the highest-affinity variant differed from b12 at every targeted position but R32, which was absolutely conserved in all the clones sequenced (90). The L1 sequence of the highest-affinity Fab is identical to that of H31L42, its whole-IgG counterpart (37) (Fig. 1). Note, however, that the multiple substitutions in CDR L1 of H31L42 occurred in the background of additional substitutions in H3 and H1, and the effects of most of these substitutions on affinity for gp120 were nonadditive (90).

A footprint of the putative contact residues of b12 on the deglycosylated core of gp120 (39), according to our docking model (66), is shown in Fig. 7E. The b12 residues whose substitution diminished gp120 binding by $\geq 95\%$ relative to the

wild type are indicated by asterisk. A useful guide in orienting the two molecules is the insertion of the "fingers" H3 (W100) and H2 (Y53) of b12 into the CD4 hydrophobic pocket and into a gap between T373 and N386 in gp120, respectively. The diminished gp120 binding of mutants W100A, Y98A, and Y53G are supportive of and supplement the model.

We postulate that W100 and Y53 contribute to the binding energy of b12 to gp120 largely by burying into a hydrophobic pocket and canyon, respectively, on either side of a ridge including S365 and D368 on gp120. Y98 of b12 is also very close to the S365-D368 ridge, which has been shown to be important for b12 binding by mutational analysis (55). Residues R29 and R32 in L1 are in close proximity to residues N276/K282 and N280/A281 in the D-loop of gp120, respectively. A recent mutational analysis of gp120 shows that substitutions N276A, K282A, and N280A slightly enhance, diminish, and have no effect on the binding of b12 to gp120, respectively (55).

From the crystal structure of b12, we calculated that the side chain of R32 was only partially accessible to solvent (Fig. 1), suggesting that the observed effect of the R32Y substitution may be due more to changes in local paratope structure than to direct contact with gp120. By contrast, the side chain of R29 is mostly accessible to solvent, suggesting that this residue might contact gp120. Alternatively, long-range electrostatic effects due to the cluster of basic residues in L1 might play a significant role in the observed effects caused by replacing any one of these b12 residues. N56 of b12 docks in close proximity to K362 of gp120; however, the N56 side chain points away from the hydrophobic canyon into which Y53 is situated, potentially explaining the absence of an effect on gp120 binding of substituting this residue. We would also add the caveat that some Ala substitutions can be energetically neutral in receptor-ligand interactions, despite replacing contact residues, as found for example in the Fab D1.3-hen egg white lysozyme complex (18). Exactly how the b12 residues discussed above spatially relate to residues in gp120 will require determination of a crystal structure of b12 in complex with gp120.

Despite the extreme differences between gp120 (≈ 120 kDa, heavily glycosylated protein) and the B2.1 peptide (dimer ≈ 4.3 kDa, nonglycosylated peptide), there are many similarities in the effects of b12 mutations on its binding to these antigens: 36 mutations had qualitatively similar effects, whereas 14 had different effects. A recently solved crystal structure of Fab b12 in complex with the B2.1 peptide should help in identifying which of these residues represent differential contacts for B2.1

FIG. 7. Molecular features of the paratope of b12. (A) Tube diagram (36) of the combining site of Fab b12, showing the position of the protruding H3 loop relative to the other CDRs. Residues in H3 for which replacement by Ala resulted in a $\geq 95\%$ decrease in apparent affinity to gp120 relative to wild-type (w.t.) b12 are labeled. (B) Stereo diagram of a ball-and-stick representation (24, 36) of the H3 loop of b12. The key residues that were labeled in A are labeled in red. (C and D) Molecular surface rendering (64, 65) of the b12 paratope (C, side view; D, top view). The light chain is colored pink, and the heavy chain is colored yellow. Residues that upon substitution caused a $\geq 95\%$ decrease in apparent affinity to gp120 relative to wild-type b12 are indicated (solid outline). Note that residue Y100i is buried. Residues S100c, P100d, A93, and S94 (dotted outline) are shown for facile comparison with panel E. The b12 structure is taken from Saphire et al. (66). (E) Crystal structure of the gp120 core (39) with the residues of b12 that are predicted from the docking model (66) to be in close proximity to the outlined region on gp120. Thus, the labeled residues are those of b12, not gp120. Putative footprints in pink and yellow are from light- and heavy-chain residues, respectively. Asterisks (*) indicate the predicted contact residues that were also found to be critical for gp120 recognition by mutational analysis in this study, as defined in panels C and D. Note: a crystal structure of b12 in complex with core gp120 is as yet unavailable. (F) Sequence conservation map of core gp120 as defined by Kwong et al. (39). Residues in blue are conserved among all primate retroviruses, residues in green are conserved among all HIV-1 isolates, residues in yellow are moderately conserved among all HIV-1 isolates, and residues in grey are variable. Carbohydrate has been modeled onto the core structures (39) of gp120.

and gp120 (E. O. Saphire, M. Montero, A. Menendez, M. B. Irving, M. B. Zwick, P. W. H. I. Parren, D. R. Burton, J. K. Scott, and I. A. Wilson, submitted for publication). The D100fE mutation, in particular, is interesting because it is conservative yet it severely impairs b12 binding to B2.1, whereas gp120 binding is almost unchanged. In addition, mutations D100aA, D100bA, 3D3A, and 3D2N all enhance b12 binding to B2.1 but reduce binding to gp120, suggesting that the "acidic patch" on the H3 loop (66) is more favorable for binding to gp120 than to B2.1. It is also noteworthy that binding of the W100 mutants to the B2.1 peptide was the same as wild-type b12, which may be expected since B2.1 does not have a deep hydrophobic cavity like gp120.

IgG1 b12 is one of a very few antibodies that exhibit potent cross-isolate anti-HIV-1 neutralizing activity (7, 8, 11, 21, 37, 79). It is therefore instructive to gather structural and functional information with respect to the neutralizing activity of IgG1 b12 in the hope of learning how to reproducibly elicit b12-like antibodies. From the antibody perspective, a question that surfaces in regard to this goal is how close to b12 does an antibody need to be in order to have the same ability to neutralize HIV-1. Two possibilities may exist: b12 is a unique specificity that cannot be reproduced with antibodies with low sequence homology to b12, or the specificity of b12 may be reproduced by a wide spectrum of nonhomologous antibodies.

Obviously, the former possibility would present a much greater challenge to template-driven vaccine design in that only anti-CD4bs antibodies that use the same germ line genes as b12 and have the correct critical residues in the CDRs would be broadly HIV-1 neutralizing. Some of these crucial residues are encoded by non-germ line DNA sequences. However, one of the very hallmarks of the humoral immune response is its ability to devise novel solutions to biomolecular recognition with molecules that have low sequence homology (i.e., the variable regions of antibodies) yet can still recognize the same epitope. Such plasticity in antigen recognition has been observed in other ligand-receptor systems (84). Clearly, additional broadly neutralizing anti-CD4bs antibodies are sorely needed in order to determine whether or not the key molecular features of b12, such as the long and rigid CDR H3 finger, the positively charged shelf-like structure in L1, and the high level of somatic mutation in b12, are required of other anti-CD4bs antibodies in order to be as broadly neutralizing against HIV-1.

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Molecular Features of the Broadly Neutralizing Immunoglobulin G1 b12 Required for Recognition of Human Immunodeficiency Virus Type 1 gp120

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IgG1 b12 is a broadly neutralizing antibody against human immunodeficiency virus type 1 (HIV-1). The epitope recognized by b12 overlaps the CD4 receptor-binding site (CD4bs) on gp120 and has been a target for vaccine design. Determination of the three-dimensional structure of immunoglobulin G1 (IgG1) b12 allowed modeling of the b12-gp120 interaction in which the protruding third complementarity-determining region (CDR) of the heavy chain (H3) was crucial for antibody binding. In the present study, extensive mutational analysis of the antigen-binding site of Fab b12 was carried out to investigate the validity of the model and to identify residues important for gp120 recognition and, by inference, key to the anti-HIV-1 activity of IgG1 b12. In all, 50 mutations were tested: 40 in H3, 4 each in H2 and L1, and 2 in L3. The results suggest that the interaction of gp120 with H3 of b12 is crucially dependent not only on a Trp residue at the apex of the H3 loop but also on a number of residues at the base of the loop. The arrangement of these residues, including aromatic side chains and side chains that hydrogen bond across the base of the loop, may rigidify H3 for penetration of the recessed CD4-binding cavity. The results further emphasize the importance to gp120 binding of a Tyr residue at the apex of the H2 loop that forms a second finger-like structure and a number of Arg residues in L1 that form a positively charged, shelf-like structure. In general, the data are consistent with the b12-gp120 interaction model previously proposed. At the gene level, somatic mutation is seen to be crucial for the generation of many of the structural features described. The Fab b12 mutants were also tested against the b12 epitope-mimic peptide B2.1, and the reactivity profile had many similarities but also significant differences from that observed for gp120. The paratope map of b12 may facilitate the design of molecules that are able to elicit b12-like activities.

It is of fundamental importance to the global human immunodeficiency virus type 1 (HIV-1) vaccine effort to look for potential ways in which to elicit an effective neutralizing antibody response against HIV-1 (6, 8, 10, 27, 32, 53, 68, 88, 92). The target of neutralizing antibodies against HIV-1 is the envelope spike, which consists of the surface glycoprotein gp120 and the transmembrane protein gp41. Although not formally proven, it is generally accepted that the spike is a trimer of gp120-gp41 heterodimers (12, 13, 33, 40, 60, 83, 87). One of the consequences of this quaternary arrangement is that a number of conserved epitopes that are well exposed on purified, monomeric gp120 and gp41 are buried or partially buried in the trimeric gp120-gp120, gp41-gp41, or gp120-gp41 interfaces within the native spike (29, 62, 67, 69, 86). The relative inaccessibility of conserved epitopes in the trimeric spike likely explains the paucity of neutralizing monoclonal antibodies against HIV-1 (8) as well as the low titers of isolate cross-neutralizing antibodies typically found in the serum of animals or humans immunized with soluble envelope protein (15, 20,

21, 23, 26, 45, 72, 81, 85) or even during natural infection with HIV-1 (38, 48).

Nevertheless, a few broadly neutralizing antibodies against HIV-1 have been described. Immunoglobulin G1 (IgG1) b12 binds to the CD4 receptor binding site (CD4bs) on gp120 (4, 9, 11, 62), 2G12 binds to a carbohydrate-rich epitope on the silent face of gp120 (63, 70, 80), and 2F5 binds to a linear epitope close to the membrane on the ectodomain of gp41 (52, 61, 94). In addition, three novel antibodies have recently been identified as having broad neutralizing activity: Fab X5, which binds an epitope on gp120, the exposure of which is enhanced by CD4 binding (51), 4E10 (74, 94), and Z13 (94), which bind immediately C-terminal to 2F5 on gp41. These antibodies stand out among the population of known human antibodies as being relatively potent and able to neutralize a wide range of primary isolates of HIV-1 (21, 51, 94) and in combination have been shown to neutralize HIV-1 with some degree of synergy (43, 95). Moreover, IgG1 b12 has recently been shown to be effective at neutralizing primary isolates of subtype C, which is responsible for the greatest number of infections worldwide (7). Importantly, IgG1 b12 is able to completely protect macaques against vaginal challenge with the simian immunodeficiency virus-HIV hybrid SHIV_{162P4} (58). This study, together with other passive antibody protection studies (2, 14, 44, 46, 56, 73), establishes parameters by which antibody can mediate

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sterile protection against retroviral challenge and illustrates the potential of broadly neutralizing antibodies for controlling HIV-1, at least in animal models.

Of the panel of broadly neutralizing anti-HIV-1 monoclonal antibodies, IgG1 b12 is the best characterized at the molecular level. Somatic variants of b12 were available essentially since its discovery by phage display methodology, providing an early indication of residues in the antibody variable regions that influence binding activity (4, 9, 57). Later, *in vitro* selection experiments were performed in which a complementarity determining region (CDR) walking strategy was used to identify variants of b12 with greater affinity for monomeric gp120 and, in some cases, with an enhanced ability to neutralize HIV-1 (5, 90). Recently, the three-dimensional structure of the whole IgG1 b12 molecule was determined (66), providing a structural framework for attempts to elucidate its broadly neutralizing activity. In particular, a Trp residue displayed at the apex of a long protruding H3 loop may allow b12 to penetrate and fill the hydrophobic cavity of the CD4bs on gp120 in a way analogous to Phe43 of CD4 (66). From a docking model of b12 with the core of gp120 (39), b12 also makes contacts on the inside face of the V1/V2 loop stem of gp120 and with the D loop of gp120 by a canyon created by CDRs H3, L1, and L3 (66). This model can now be tested by additional structural and functional studies.

IgG1 b12 is also arguably the best characterized of a group of antibodies known as anti-CD4bs antibodies, which compete with CD4 and with each other in binding to gp120. Many human anti-CD4bs antibodies (besides IgG1 b12) have been described by various groups (49, 50, 75), including 15e (30), F105 (76), F91 (50), 1125H (77), 21h (30, 75), 654-30D (41), and Fab b6 (57, 62), the last of which was isolated from the same seropositive subject from whom b12 was cloned. These anti-CD4bs antibodies often show broad reactivity with monomeric gp120s from different isolates of HIV-1 but do not, however, show the neutralizing activity of b12 (21, 22, 62). The difference has been associated with the ability of b12 but not of other anti-CD4bs antibodies to bind well to the trimeric envelope spike on the surface of virions (59). It would seem that b12 is able to bind with reasonably high affinity to both monomeric and trimeric forms of gp120, whereas the other CD4bs antibodies bind well only to the monomeric form.

Clearly, one would like to have immunogens capable of eliciting b12-like antibodies. To this end, we have been exploring the interaction of b12 with gp120 from a number of aspects. These include determination of the crystal structure of b12 (66), docking of b12 with the structure of the core of gp120 (66), and examination of the effects of mutations of gp120 residues on the b12-gp120 interaction (55). Here, we approached the problem from the point of view of the antibody by attempting to identify the key structural features of b12 required for gp120 binding through extensive mutagenesis of b12 residues. At the same time, for comparative purposes, we looked at the effects of mutations on the interaction of b12 with a peptide mimotope, B2.1, that binds b12 specifically and is being studied as a vaccine lead (93). The results provide functional data relevant to the docking model presented previously (66) and reveal specific requirements at the tip and base of the CDR H3 finger of b12 for gp120 recognition. In addition, a cluster of arginine residues in the CDR L1 region

forming a shelf-like structure and a prominently displayed Tyr residue in CDR H2 are shown to be crucial. The potential implications for eliciting b12-like antibodies by vaccination are discussed in terms of the demands that this puts on the antibody repertoire.

MATERIALS AND METHODS

Mutagenesis and crude Fab preparation. b12 Fab mutants were engineered with the QuikChange mutagenesis kit (Stratagene) according to the manufacturer's directions with pComb3H vector DNA, encoding wild-type b12 Fab, as the template. A similar approach was used to engineer the Fab b6 mutants. The sequences of the mutant clones were verified by DNA sequencing within the variable regions. A complete list of the Fab b12 mutants engineered in this study is included in Fig. 1. The CDRs were defined with IMGT delimitations (<http://imgt.cnusc.fr:8104/home.html>) (42) except for H3, for which conserved residues A93 and R94 were omitted, as per the Kabat and Wu definition (34).

The preparation of crude Fab supernatants has been described previously (3). Briefly, the mutant clones, wild-type b12, and an irrelevant Fab negative control were transformed separately into *Escherichia coli* XL1-Blue cells (Stratagene), and single colonies were used to inoculate 10-ml cultures in SB medium containing 50 µg of carbenicillin and 10 µg of tetracycline per ml. The cultures were shaken at 300 rpm at 37°C for 6 to 8 h, then induced with 1 mM isopropylthiogalactopyranoside (IPTG), and incubated overnight at 30°C with shaking. The next day, the cultures were centrifuged at 5,000 × g for 15 min at 4°C, the pellets were resuspended in 1 ml of phosphate-buffered saline (PBS, pH 7.0), and the bacterial suspensions were subjected to four rounds of freeze-thawing. The bacterial debris was pelleted at 14,000 rpm in a microcentrifuge, and the supernatants were supplemented with bovine serum albumin (BSA) and Tween 20 (1% and 0.025% final concentrations, respectively). Duplicate or triplicate crude Fab supernatants were prepared to lessen the effect of culture-to-culture variation in Fab production, pooled, and used directly for enzyme-linked immunosorbent assays (ELISAs) as described below.

Crude Fab ELISA. Ninety-six-well plates (one-half diameter, flat-bottomed; Costar) were coated with 50 µl of PBS containing 50 ng of goat anti-human IgG F(ab')₂ (Pierce), 75 ng of gp120_{RFL} (Progenics), 50 ng of oligomeric gp120_{IIIIB} (ImmunoDiagnostics, Inc.), or 7 × 10⁹ B2.1 phage particles (the B2.1 peptide HERSYMFSDLENRCI is a disulfide-bridged, homodimeric peptide displayed as a fusion to the N terminus of pVIII on the filamentous phage [93]) and incubated overnight at 4°C. The wells were washed twice with PBS containing 0.05% Tween 20 and blocked with 3% BSA at 37°C for 1 h. The wells were washed once, and 50 µl of the bacterial supernatants containing Fab diluted in PBS containing 1% BSA and 0.025% Tween 20 was added. The plates were incubated for 2 h at 37°C, the wells were washed four times, goat anti-human Fab conjugated to alkaline phosphatase (Pierce), diluted 1:500 in PBS containing 1% BSA and 0.025% Tween 20, was added to the wells, and the plate was incubated at room temperature for 30 min. The wells were washed five times and developed by adding 50 µl of alkaline phosphatase substrate, prepared by adding one tablet of disodium *p*-nitrophenyl phosphate (Sigma) to 5 ml of alkaline phosphatase staining buffer (pH 9.8), according to the manufacturer's instructions.

After ~30 min, the optical density at 405 nm was read on a microplate reader (Molecular Devices). The concentration of Fab was determined with the anti-Fab ELISA (full curve, threefold dilution series) with simple linear regression; the concentrations of Fab in the samples were usually within about twofold of that of wild-type Fab b12 except for mutants V95A, Y53G, 3D3A, and 3D3N, which were consistently 4- to 10-fold less abundant. A full threefold ELISA binding curve was also generated for groups of Fab mutants alongside wild-type b12 and a negative Fab control against gp120 or B2.1 phage. Apparent affinities were calculated as the antibody concentration at half-maximal binding. Apparent affinities as a percentage of that of wild-type Fab b12 were calculated with the formula [(apparent affinity of the wild type)/(apparent affinity of the mutant)] × 100. All samples were tested at least twice, and the mean was taken as the final reported value.

Competition ELISA with purified Fab. Ninety-six-well plates were coated with gp120, washed, and blocked, as above. Wild-type and representative mutants of Fab b12 were purified as described previously (3) with protein G-Sepharose columns (Fast Flow; Pharmacia) and verified to be >90% pure by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The purified Fabs were added to the wells at various concentrations in the presence of a single concentration of biotinylated Fab b12 that was previously determined to generate an ELISA signal of 50 to 75% of maximal. After 2 h of incubation at 37°C, the plate was washed, and a streptavidin-horseradish peroxidase conjugate (Jack-

A	Clone ID	CDR H3
b12 (w.t.)	VGPYSWDDSPQDNYYMDV	
Solvent access#	0 0 3 2 2 4 3 1 3 3 1 2 0 1 0 0 1 1	
V95A	A - - - - - - - - - - - - - - -	
V95I	I - - - - - - - - - - - - - - -	
G96A	- A - - - - - - - - - - - - - - -	
P97A	- - A - - - - - - - - - - - - - - -	
P97E	- - E - - - - - - - - - - - - - - -	
Y98A	- - - A - - - - - - - - - - - - - - -	
Y98W	- - - W - - - - - - - - - - - - - - -	
S99A	- - - - A - - - - - - - - - - - - - - -	
S99G	- - - - G - - - - - - - - - - - - - - -	
W100A	- - - - - A - - - - - - - - - - - - - - -	
W100F	- - - - - F - - - - - - - - - - - - - - -	
W100S	- - - - S - - - - - - - - - - - - - - -	
W100V	- - - - V - - - - - - - - - - - - - - -	
D100aA	- - - - - A - - - - - - - - - - - - - - -	
D100aE	- - - - - E - - - - - - - - - - - - - - -	
D100bA	- - - - - A - - - - - - - - - - - - - - -	
D100bE	- - - - - E - - - - - - - - - - - - - - -	
S100cA	- - - - - A - - - - - - - - - - - - - - -	
P100dA	- - - - - A - - - - - - - - - - - - - - -	
Q100eA	- - - - - A - - - - - - - - - - - - - - -	
Q100eN	- - - - - N - - - - - - - - - - - - - - -	
Q100eF	- - - - - F - - - - - - - - - - - - - - -	
D100fA	- - - - - A - - - - - - - - - - - - - - -	
D100fE	- - - - - E - - - - - - - - - - - - - - -	
N100gA	- - - - - A - - - - - - - - - - - - - - -	
N100gD	- - - - - D - - - - - - - - - - - - - - -	
N100gQ	- - - - - Q - - - - - - - - - - - - - - -	
N100gH	- - - - - H - - - - - - - - - - - - - - -	
N100gY	- - - - - Y - - - - - - - - - - - - - - -	
Y100hA	- - - - - A - - - - - - - - - - - - - - -	
Y100hF	- - - - - F - - - - - - - - - - - - - - -	
Y100hW	- - - - - W - - - - - - - - - - - - - - -	
Y100iA	- - - - - A - - - - - - - - - - - - - - -	
Y100iF	- - - - - F - - - - - - - - - - - - - - -	
Y100iW	- - - - - W - - - - - - - - - - - - - - -	
M100jA	- - - - - A - - - - - - - - - - - - - - -	
D101A	- - - - - A - - - - - - - - - - - - - - -	
V102A	- - - - - A - - - - - - - - - - - - - - -	
3D3A	- - - - - AA - - - - - A - - - - - A - - - - -	
3D2N	- - - - - NN - - - - - A - - - - - A - - - - -	
b7*	- - - T - - - - - - - - - - - - - - -	
MV2*	- - E - K - - N - - - - - - - - - - -	
3B1**	- - QWN - - - - - - - - - - - - - - -	
3B2**	- - WT - - - - - - - - - - - - - - -	
3B3**	- - EWG - - - - - - - - - - - - - - -	
3B4**	- - WN - - - - - - - - - - - - - - -	
3B6**	- - LWN - - - - - - - - - - - - - - -	
3B7**	- - SWR - - - - - - - - - - - - - - -	
3B9**	- - WR - - - - - - - - - - - - - - -	
H1.3B/H3.32**	- EWG - EQFRF - - - - - - - - - - -	
H1.3B/H3.33**	- EWG - EMFRY - - - - - - - - - - -	
H1.3B/H3.34**	- EWG - EMRRF - - - - - - - - - - -	
H1.3B/H3.35**	- EWG - HQRRY - - - - - - - - - - -	
H1.3B/H3.36**	- EWG - QRRY - - - - - - - - - - -	
H1.3B/H3.38**	- EWG - TQRRF - - - - - - - - - - -	
H1.3B/H3.39**	- EWG - QVRY - - - - - - - - - - -	
CS**	- EWT - - - - - - - - - - - - - - -	
CS**	- EWT - - F - - - - - - - - - - -	
CS**	- EWT - MD - - A - - - - - - - - - -	
B	Clon ID	CDR H1
b12 (w.t.)	GYRFSNFV	
Solvent access#	2 0 3 0 2 1 1 0	
3B3#	- - - - - T	
H31L42#	- - - - H - T	
"germline"	- - T - T SYA	
C	Clone ID	CDR H2
b12 (w.t.)	INPYNGNK	
Solvent access#	0 1 0 3 2 3 3 2	
N52A	- A - - - - -	
P52aA	- - A - - - -	
Y53G	- - - G - - -	
N56A	- - - - - A -	
"germline"	- - A G - - - T	
D	Clone ID	CDR L1
b12 (w.t.)	HSIRSR	
Solvent access#	2 2 0 3 2 2 1	
R29S	- - - S - - -	
S30A	- - - - A - -	
R31S	- - - - - S -	
R32Y	- - - - - Y	
b21*	- N - - - - -	
H31L42**	- QL DGS -	
"germline"	Q - VS - SY	
E	Clone ID	CDR L2
b12 (w.t.)	G V S	
Solvent access#	1 1 2	
"germline"	- A -	
F	Clone ID	CDR L3
b12 (w.t.)	QVYGASSYT	
Solvent access#	0 0 0 0 3 3 1 0 1	
A93Y	- - - Y - - -	
S94A	- - - - A - -	
b7*	- Q - - S - R -	
H31L42**	- Q - - WP F - -	

FIG. 1. Amino acid sequences of mutants and variants of wild-type (w.t.) b12 in CDR loops H3 (A), H1 (B), H2 (C), L1 (D), L2 (E), and L3 (F). Each panel shows the solvent accessibility (Solvent access#) of the side chains of each residue, the relevant sequences of mutants of b12 engineered for this study, and natural (*) and in vitro-evolved (**) variants isolated in previous studies (references 4 and 57 and references 5 and 90, respectively). The solvent accessibilities of the side chains were determined by the NACCESS computer program (16) and are graded as follows: 0, buried (<10% exposure); 1, partially exposed (10 to 35% exposure); 2, moderately exposed (36 to 66% exposure); 3, mostly exposed (67 to 90% exposure); and 4, fully exposed (>90% exposure). H31L42 (37) is a whole-IgG version of the Fab designated h1.1 h3.33/L1.4L3.14 (90). The deduced amino acid sequences of the closest related germ line DNA are shown for comparison; the germ line sequences for the heavy and light chains are IGHV1-3*01 (DP-25; accession no. X62109) and IGVK3-20*01 (DPK22; accession no. X12686), respectively.

son) diluted 1:1,000 in PBS containing 1% BSA and 0.025% Tween 20 was added. After a 30-min incubation at room temperature, the plates were washed, and the tetramethylbenzidine (TMB) substrate kit (Pierce) was used for developing, according to the manufacturer's instructions. The optical density at 450 nm was read on a microplate reader (Molecular Devices), and the results were recorded as the 50% inhibitory concentration (IC_{50}), defined as the concentration of competing Fab that was required to reduce the maximal signal generated by biotinylated Fab b12 by 50%. All competition ELISAs were performed twice.

Synthesis of b12 CDR H3 peptide and coupling to BSA. A peptide corresponding to CDR H3 of b12 was synthesized with the b12 crystal structure as a guide (66); the peptide has the sequence C-PGK-A⁹³RVGPYSWDDSPQD NYYMDW¹⁰³. Residues PGK were included to promote a type II β -turn (31), and a Cys residue was added to facilitate cyclization via native chemical ligation (19) and chemical coupling to a carrier molecule. The Arg 94 side chain in the third framework region (FR3) projects into solvent in the crystal structure and so was included in the peptide, as was Ala 93. The peptide was synthesized with *N*-tert-butoxy-carbonyl chemistry (71), purified by reverse-phase high-pressure liquid chromatography to >95% purity, and verified by mass spectrometry.

To cyclize the peptide, the backbone was linked N to C terminus by native chemical ligation (28), and the final product was purified by reverse-phase high-pressure liquid chromatography to >95% purity and verified by mass spectrometry. The free Cys of the peptide was used to cross-link the peptide to activated BSA (Pierce) by using the manufacturer's instructions. Briefly, 10 mg of sulfo-succinimidyl 4-(*N*-maleimidomethyl)cyclohexane-1-carboxylate-activated BSA in 1 ml of deionized H₂O was mixed with 10 mg of peptide dissolved in 0.25 ml of dimethyl sulfoxide and 1.75 ml of PBS. The mixture was rocked gently for 2 h at room temperature, dialyzed extensively against PBS, and then sterilized with a 0.2- μ m filter. A control peptide (GTP-binding peptide of sequence CEGN-VRSRELAGHTGY; American Peptide Co.) was similarly linked to BSA. The protein concentration of each sample was determined with the Micro BCA protein assay kit (Pierce) according to the manufacturer's instructions. The conjugates were also analyzed by SDS-PAGE, and cross-linking was confirmed by an increase in the molecular weight of the activated BSA after reacting with the peptide; both the b12 CDR H3 and the control peptide caused a similar shift in molecular weight.

HIV-1 neutralization assay. The primary isolate HIV-1_{JRFL} was assayed for neutralization with peripheral blood mononuclear cells as target cells and detection of p24 in ELISA as a reporter assay, as described previously (94).

Nucleotide sequence accession number. The sequences of the heavy and light chains of b12 have been deposited in GenBank (accession no. AAB26315.1 and AAB26306.1, respectively).

RESULTS

Alanine-scanning mutagenesis of CDR H3 of b12. In the mutagenesis strategy, we chose to compare binding of different mutants of b12 to gp120 with crude Fab supernatants prepared from bacterial cultures (3), which facilitated the rapid analysis of a large number of Fab mutants (Fig. 1) that otherwise would not have been feasible. Apparent affinities relative to wild-type Fab b12 were measured by ELISA, as described in Materials and Methods. As a first step in our analysis of b12, we performed a complete alanine scan of the CDR H3 loop and tested the mutant Fabs by ELISA to determine their relative binding strengths to gp120. We chose recombinant gp120 from two different strains, one from a primary isolate (gp120_{JRFL}) and one from a T-cell-line-adapted strain (gp120_{IIIB}), to see whether any differences in binding could be found with our mutant Fab panel. We also included in our analysis the previously described, b12-specific peptide B2.1, as displayed on filamentous phage (93). The dissociation constant (K_d) of wild-type Fab b12 against gp120_{LAI} is ≈ 9 nM (62), against the B2.1 synthetic peptide is ≈ 2.5 μ M (93), and against B2.1 phage is closer to the K_d of the b12-gp120 interaction because of the constraint that the phage coat imparts to the peptide, although

a precise K_d value of b12 for the phage-associated peptide is difficult to determine (93).

The results of the alanine scan of the H3 loop of b12 are shown in Fig. 2. Substitution of 7 out of 18 residues in H3 with Ala reduced binding of Fab b12 to both gp120_{JRFL} and gp120_{IIIB} by greater than 90%. The relevant mutants are V95A, Y98A, W100A, Q100eA, N100gA, Y100hA, and Y100iA. [The CDR H3 of b12 contains a 10-residue insertion. In Kabat and Wu numbering (34), these residues are designated 100A, 100B, . . . , 100J. For clarity, these inserted residues will be denoted with a lowercase letter after the number of the residue position so as to avoid confusion with the mutated residue (uppercase) when referring to mutations (e.g., mutation D100aA)]. The loss of antigen binding of the W100A mutant was not unexpected from the crystal structure of IgG1 b12 and a docking model of b12 and the gp120 core (66). However, the behavior of some of the other mutants was more surprising.

Substitutions in b12 that reduced gp120 binding as much as or even more than observed with the W100A mutant included V95A, Y98A, Q100eA, N100gA, Y100hA, and Y100iA. Most of these substitutions are at the base of the CDR H3 and involve side chains that are poorly exposed to solvent, as defined in Fig. 1. Solvent accessibility to side chain (Fig. 1) was not, however, predictive of the effect of a substitution on antigen binding (Fig. 2). The greatest effects of an Ala substitution on gp120 recognition occurred with residues N100g, Y100h, and Y100i at the C-terminal end of H3, which resulted in complete loss of gp120 recognition in our assay (<0.1% apparent affinity relative to wild-type b12). We confirmed these results by purifying Fab protein for these mutants and were unable to observe significant reactivity with gp120 even at 20 μ g/ml (data not shown). To further explore these findings, additional substitutions were made in these positions and tested for binding to gp120 and the B2.1 peptide (see below). Significantly, there was generally good agreement in the relative binding of Ala mutants to gp120_{JRFL} (primary isolate) and gp120_{IIIB} (T-cell-line-adapted).

Thirteen Ala substitutions in H3 had qualitatively similar effects on the ability of b12 to bind to gp120 and B2.1, suggesting some similarity in recognition of these antigens by b12. However, the effects of other Fab substitutions such as Y98A, W100A, D100aA, and D100bA did not correlate, pointing to possible ways of improving the B2.1 peptide as a b12 epitope mimic.

Mutations in CDR H3 W100. A striking feature in the crystal structure of IgG1 b12 is the prominent display of the aromatic residue W100 at the apex of the long H3 loop (66). As shown in Fig. 2 and 3A, Ala substitution at W100 significantly diminished binding of Fab b12 to gp120_{JRFL} and gp120_{IIIB}. This result was confirmed with purified Fab: the W100A mutant inhibited biotinylated Fab-b12 with an IC_{50} of 29 μ g/ml and 50 μ g/ml for gp120_{JRFL} and gp120_{IIIB}, respectively, compared to an IC_{50} of 3 μ g/ml and 1.5 μ g/ml, respectively, for wild-type Fab b12 (data not shown).

In the b12-gp120 docking model, W100 was acting to fill the hydrophobic cavity of the CD4bs on gp120 in a way analogous to F43 of CD4. Thus, we also made the W100F mutant to determine whether Phe would be more or less favored than Trp at this position. Retention of an aromatic ring in the

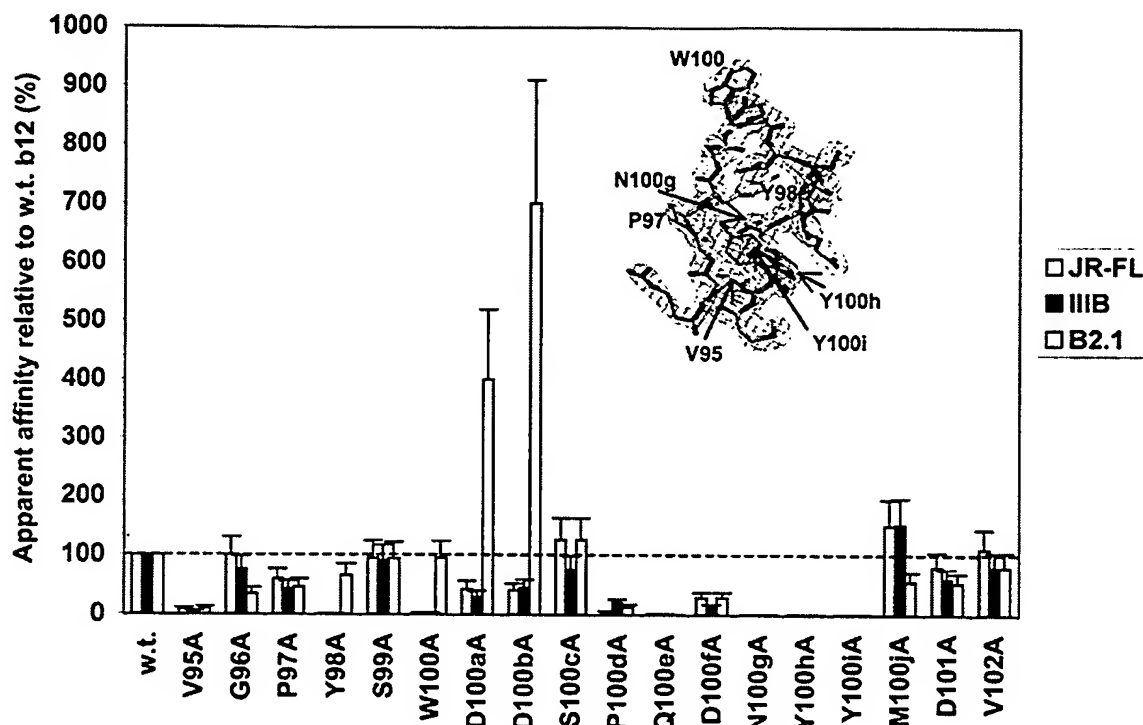


FIG. 2. Alanine-scanning mutagenesis of CDR H3 of b12. Bars indicate the apparent affinities of Fab mutants relative to wild-type (w.t.) Fab b12 for gp120_{JRFL}, gp120_{IIIB}, and the B2.1 peptide. The H3 loop and corresponding electron density from the intact b12 structure (66) are shown (inset), with key residues indicated.

W100F mutant did promote slightly better binding than the W100A mutant to both gp120s, but the apparent affinity was $\approx 10\%$ relative to that of the wild type. Two other mutants, W100S and W100V, also had considerably impaired binding to both gp120s. Taken together, these results confirm the importance of W100 for b12 by showing that Trp is preferred over four other residues, including another aromatic, Phe, at this position. In contrast to the results with the gp120s, the W100 mutants were all able to bind B2.1 at nearly wild-type levels, strongly suggesting that W100 is not involved in B2.1 recognition.

Role of Asp residues in CDR H3 of b12. Another feature of the b12 crystal structure that initially drew our attention was a clustered grouping of acidic moieties on one face of the H3 loop (66). We wondered whether this "acidic patch" was involved in keeping the H3 loop of b12 erect via charge repulsion with a weakly acidic patch near the base of the H3 loop. The residues involved included D100a, D100b, and D100f, as well as hydroxyl groups from Ser 99, Ser 100c, and Tyr 100i (D101 was not involved in this patch and moreover was found to have little involvement in binding to gp120 and B2.1; see Fig. 2). The Ala substitutions (Fig. 2) had mostly moderate effects on binding to gp120 (≈ 2 - to 3-fold-reduced binding relative to wild-type b12). Further mutagenesis (Fig. 3B) yielded mutants D100aE, D100bE, and D100fE, the substitutions in which either had no effect (D100aE) or again only moderate effects (D100bE and D100fE) on binding to gp120. Two additional mutants were constructed in which all three positions were changed to see if there was any cooperativity among these

residues. A triple Ala mutant, dubbed 3D3A, and another triple mutant, D100aN/D100bN/D100fA, dubbed 3D2N, were still able to consistently bind to gp120_{JRFL} and gp120_{IIIB}, albeit at somewhat lower levels (data not shown).

The acidic patch on the paratope of b12 appears to have significance for B2.1 recognition. The Ala mutants D100aA, D100bA, and both triple mutants bound to B2.1 much better than to wild-type Fab b12. Interestingly, the D100fE mutant bound extremely poorly to B2.1 but bound gp120 almost as well as the wild type. In fact, the D100fE mutant bound more poorly to B2.1 than the "less conservative" D100fA mutant, revealing a very distinct requirement for B2.1 recognition at position 100f.

Mutagenesis in CDR H3 of b12 N-terminal to W100. Four residues N-terminal to W100 in H3 of b12 were chosen for additional study. V95 was chosen because the Ala mutation at this position caused severe impairment in binding to gp120, and we wished to determine whether a more conservative substitution with Ile would also reduce binding. Figure 3C shows that the V95I mutant bound gp120 at nearly wild-type levels. The extra bulk of a branched aliphatic side chain at this position is probably necessary for full activity of b12. The residues P97, Y98, and S99 were also targeted for further mutagenesis, inspired by previously published variants of b12 (both naturally occurring and in vitro-enhanced variants), including 3B3, in which the sequence EWG is found in place of PYS at these positions (4, 5) (Fig. 1A). The Y98W mutant indeed bound ≈ 3 - to 9-fold better to the gp120s than did wild-type Fab b12 (Fig. 3C). The P97E and S99G mutants

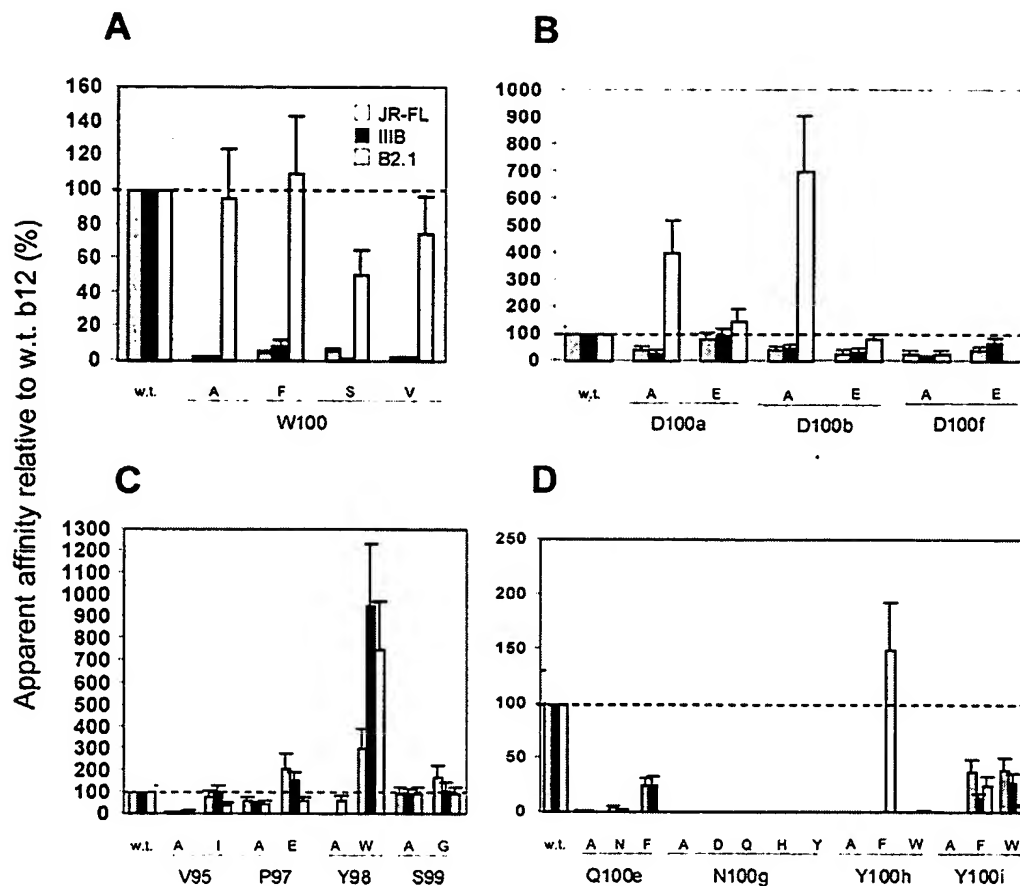


FIG. 3. Further substitution analysis of residues in the H3 loop of b12. Bars indicate the apparent affinities of Fab mutants relative to wild-type (w.t.) Fab b12 for gp120_{JRFL}, gp120_{IIIB}, and the B2.1 peptide. Substitutions were made to Trp100 (A), Asp residues near the crown of the H3 loop (B), selected H3 residues N-terminal to Trp100 (C), and key residues C-terminal to Trp100 (D).

bound to gp120 as well as or only slightly better than wild-type b12. Thus, the enhanced binding of 3B3 to gp120 presumably derives mostly from the preference for a W over a Y at the middle position of the EWG motif, with the flanking residues perhaps providing additional fine tuning.

In contrast to the Ala mutant, P97A, B2.1 binding was not correlated with gp120 binding for mutant P97E. Interestingly, the substitution Y98W enhanced the binding of Fab b12 to both B2.1 and gp120, whereas the S99G substitution was silent for both gp120 and B2.1.

Mutagenesis in CDR H3 of b12 C-terminal to W100. The alanine scan (Fig. 2) showed that residues Q100e, N100g, Y100h, and Y100i in the C-terminal portion of H3 of b12 were all important for gp120 recognition and were therefore chosen for further mutational analysis. Q100eN and Q100eF mutants also bound gp120 with diminished affinity relative to wild-type Fab b12, although the gp120 binding was significantly improved for Q100eF relative to Q100eA (Fig. 3D). For verification purposes, Fab Q100eA was purified and used in a competition ELISA with biotinylated Fab b12 against gp120_{IIIB}; the IC₅₀ of Q100eA was ≈ 14 μ g/ml, which is nine times greater than that of wild-type Fab b12 (IC₅₀ ≈ 1.5 μ g/ml) and consistent with the crude Fab ELISA. In the structure of IgG1 b12, the side chain of Q100e is only partially accessible to solvent

and makes a hydrogen bond with the main chain of A93. A loss of this hydrogen bond might destabilize the interaction at the base of CDR H3 and could explain the observed reduction in binding to gp120 for the Q100e mutants.

Next, we tested more conservative substitutions at position N100g. Neither an N100gD nor an N100gQ mutant was able to bind either gp120_{JRFL} or gp120_{IIIB}, indicating that the removal of an amino group or the addition of a methylene group, respectively, from the side chain of Asn100g was sufficient to completely abolish gp120 recognition in our assay format (Fig. 3D). In the crystal structure of b12, the amino nitrogen on the side chain of Asn100g makes a hydrogen bond with the main-chain carbonyl of Gly96; indeed, the side chain of Asn100g is $<10\%$ exposed to solvent (Fig. 1). Again, mutations in Asn100g most likely affect the structure of the paratope of b12 due to the absence of the stabilizing hydrogen bond. Thus, not surprisingly, the substitution of Asn100g with either His or Tyr completely abolished binding to either gp120_{JRFL} or gp120_{IIIB}. The Tyr substitution was chosen because the JH6 family of J segments (the J-segment family used by b12 in VDJ-recombination) encodes a repeating string of Tyr residues, and thus b12-like antibodies that also use the JH6 family might encode a Tyr at this position. The complete lack of detectable binding to gp120 by any of the 100g mutants underscores the critical

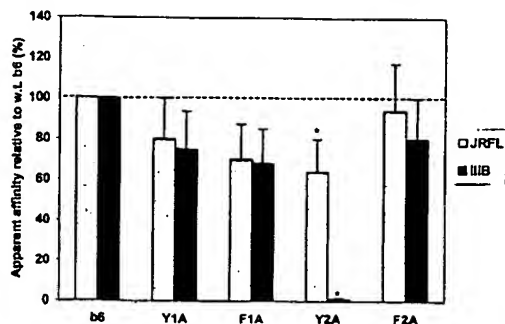


FIG. 4. Effect of alanine substitutions of the four aromatic residues in the H3 loop of b6 on relative binding of Fab b6 to gp120_{JR-FL} and gp120_{IIIB}. Bars indicate the apparent affinities of Fab mutants relative to wild-type (w.t.) Fab b6 for gp120_{JR-FL} and gp120_{IIIB}. The amino acid sequence of the H3 loop of b6 is QKPR¹Y¹F¹DL¹SGQY²RRVAGAF²DV (the aromatic residues are in boldface and have superscript numbers to correspond with the bar graph). By ELISA, the half-maximal binding of the purified Fab of the Y²A mutant to gp120_{IIIB} was 0.4 μ g/ml, which was \approx 50 times lower than the half-maximal binding of mutant Fab Y²A to gp120_{JR-FL}, 0.007 μ g/ml (*); half-maximal binding of wild-type Fab b6 to both gp120_{IIIB} and gp120_{JR-FL} was determined to be 0.003 μ g/ml (data not shown).

importance of residue N100g in the activity of b12 (see the Discussion).

In contrast to the irreplaceability of N100g, antigen recognition was found to be at least partially maintained with conservative substitutions to Y100h and Y100i. Thus, the Y100i(F/W) mutants bound to gp120 at \approx 20% to 40% wild-type levels (Fig. 3D). These results imply that a bulky aromatic at position 100i is required by Fab b12 for recognition of gp120 but that the added hydroxyl group of Tyr100h is important for full antigen binding. By contrast, only a Tyr residue appears to be sufficient for gp120 recognition at position 100h, but the Y100hF mutant was able to bind B2.1 at wild-type levels.

Aside from mutant Y100hF, all the other b12 H3 mutants (conservative mutations C-terminal to W100) had impaired binding to B2.1. Interestingly, a Phe substitution at Y100h but not at Y100i fully restored B2.1 binding. This may reflect the observation that the two Tyr residues are pointing in roughly opposite directions in the crystal structure of b12.

Substitution of aromatic residues in H3 loop of nonneutralizing anti-CD4bs antibody b6. Following our analysis of the H3 loop of b12, we were particularly interested in the role of aromatics because substitution to Ala of all four aromatics in the H3 loop of b12, not only W100, caused a $>$ 95% decrease in relative binding to gp120. Thus, we chose to see if H3 loop aromatics were important in the function of another anti-CD4bs antibody, b6, which, like b12, has a long H3 loop with four aromatic residues but does not neutralize primary isolates of HIV-1 (62). The sequence of the H3 loop of b6 is given in Fig. 4, and the aromatic residues are designated Y¹A, F¹A, Y²A, and F²A. The results (Fig. 4) indicated that in contrast to b12, mutation to Ala of only one of the four aromatics in the H3 of b6, Y²A, led to a severe decrease in binding of Fab b6 to gp120 and only to gp120_{IIIB} and not gp120_{JR-FL}. In fact, the Y²A substitution decreased the half-maximal binding of purified Fab b6 to gp120_{IIIB} from 0.003 μ g/ml to 0.4 μ g/ml, which was far more severe than the change in half-maximal binding to

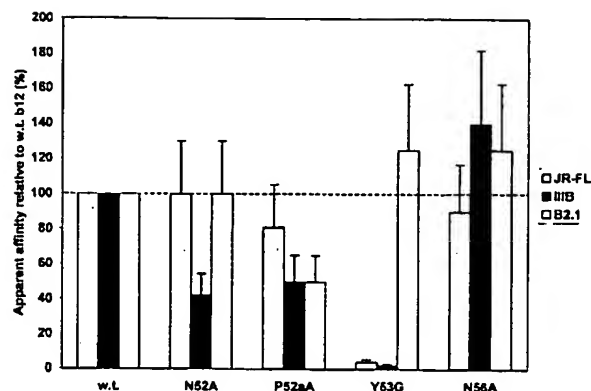


FIG. 5. Effect of substitutions in the H2 loop of b12 on relative binding of Fab b12 to gp120_{JR-FL}, gp120_{IIIB}, and the B2.1 peptide. Bars indicate the apparent affinities of Fab mutants relative to wild-type (w.t.) Fab b12 against gp120_{JR-FL}, gp120_{IIIB}, and the B2.1 peptide. The mutations P52aA and Y53G were back mutations to the residues encoded by the closest related germ line DNA (see Fig. 1).

gp120_{JR-FL} (from 0.003 μ g/ml to 0.007 μ g/ml; data not shown). The differences in the roles of H3 aromatics between b12 and the poorly neutralizing antibody b6 are striking and are discussed below.

b12 H2 mutations. In the b12-gp120 docking model (66), it was predicted that residues in H2 could contact gp120. Residue Y53 is the most prominent in H2, points directly toward gp120 in the model, and occupies a large space in the b12-gp120 interface. We suspected that residue P52a might also play a role in gp120 recognition by maintaining the H2 loop in a particular conformation rather than by making extensive contact with gp120. Interest in residues P52a and Y53 was also strong because these were nonconservatively mutated from the residue encoded by the closest germ line genes at these positions, Ala and Gly, respectively. The germ line genes closest to b12 are DPK22 and DP-25 (<http://imgt.cnusc.fr:8104/> [17, 78]) for the light and heavy chains, respectively, and the residues they encode are shown for each CDR in Fig. 1. The strategy of using germ line "back mutations" for evaluating residues outside of H3, where somatic mutation is key to generating residue diversity, was adopted to determine the dependence of b12 binding on somatic mutation. Residues N52 and N56 were also in the interface between b12 and gp120 and thus were targeted for mutagenesis.

With the exception of Y53G, the H2 substitutions had only moderate to slight effects on Fab binding to gp120_{IIIB}, gp120_{JR-FL}, and B2.1 (Fig. 5). Residue N52 was predicted to make relatively minor contact with gp120, and correspondingly the N52A substitution only moderately diminished binding against gp120_{IIIB}. Interestingly, the N56A mutant bound gp120 at wild-type levels (Fig. 5), implying that this residue does not significantly contribute to gp120 binding. Residue N56 docks in close proximity to residue K362 of gp120; however, the side chain of N56 points away from the contact surface, potentially accounting for the absence of an effect on gp120 binding of the N56A substitution. The P52aA substitution was expected to affect H2 loop structure rather than replace a contact residue, and the effect on gp120 binding was moderate (i.e., \approx 2-fold reduction in apparent affinity for gp120), indicating that P52a

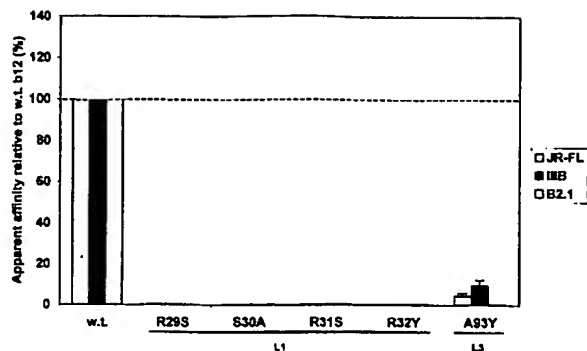


FIG. 6. Effect of substitutions in the CDR loops L1 and L3 of b12 on the relative binding of Fab b12 to gp120_{JR-FL}, gp120_{IIIB}, and the B2.1 peptide. Bars indicate the apparent affinities of Fab mutants relative to wild-type (w.t.) Fab b12 for gp120_{JR-FL}, gp120_{IIIB}, and the B2.1 peptide. The mutations R29S, R31S, and R32Y were back mutations to the residues encoded by the closest related germ line DNA (see Fig. 1).

has a rather modest role in gp120 binding. The Y53G substitution, by contrast, greatly diminished the binding of b12 to gp120, suggesting that Y53 contributes significantly to the binding energy between b12 and gp120. Residue Y53 protrudes to form a second "finger" in the paratope of b12 and was predicted to bury into a canyon in gp120 (see Discussion). The Y53G substitution had little effect on B2.1 recognition, implying that B2.1 does not contact Y53, considering that the side chain of Y53 is mostly solvent exposed (Fig. 1). We note that attempts to purify the Y53G mutant resulted in a somewhat impure Fab preparation that bound poorly to gp120 and B2.1 (data not shown), whereas the crude Fab bound B2.1 at wild-type levels, suggesting that this mutant might be unstable to the purification conditions, which involve acid elution.

b12 L1 and L3 mutations. Finally, the b12-gp120 docking model indicated that various residues in CDRs L1 and L3 could play a role in binding to gp120. Residues R29, R31, and R32 in L1 were chosen for substitution to S, S, and Y, respectively, because the closest germ line genes encode the substituted residues at these positions. S30 was changed simply to Ala because the closest germ line gene already encodes a Ser at this position. The results indicate that all four of these L1 mutants were essentially unable to recognize gp120 (Fig. 6) and suggest that somatic mutations found in this loop are essential to b12 specificity. Two mutations were made in L3 (A93Y and S94A), also based on predictions of their interaction with gp120. The A93Y mutant suffered a significant loss in gp120 binding (Fig. 6). Fab A93Y was purified and used in a competition ELISA with biotinylated Fab b12 against gp120_{IIIB}; the IC₅₀ of A93Y was $\approx 10 \mu\text{g/ml}$, which is six to seven times greater than that of wild-type Fab b12 (IC₅₀ $\approx 1.5 \mu\text{g/ml}$) and consistent with the crude Fab ELISA. Unfortunately, the S94A mutant was found to be poorly produced in crude bacterial supernatants relative to the wild type (>10 -fold reduction; data not shown), precluding a quantitative analysis of binding of the latter Fab mutant. Nevertheless, binding of the S94A Fab mutant to gp120 was detectable despite the low concentration of Fab in the supernatants, and we were able to conclude that the S94A mutation is at least not a complete knockout mutation (data not shown).

Whereas the mutations in L1 completely knocked out the binding of Fab b12 to both B2.1 and gp120 in our assay, the L3 mutation, A93Y, abolished binding to B2.1, but binding to gp120 was maintained, albeit at $\approx 10\%$ of wild-type levels (Fig. 7). It is unclear whether the residues in L1 contact gp120 or if their mutation disrupts the b12 paratope. However, it appears that A93Y is important for B2.1 recognition because the paratope of the A93Y mutant was sufficiently intact to bind to gp120 with measurable affinity (Fig. 7). As for the poorly produced Fab mutant S94A, the result was similar for B2.1 as for gp120; at most, only a moderate effect on B2.1 binding was expected for this mutation (data not shown), although a quantitative analysis was not attempted.

Neutralization of HIV-1 by synthetic CDR H3 b12 peptide.

We recently reported the neutralization of the T-cell-line-adapted strains HIV-1_{MN} and HIV-1_{IIIB} by a conjugate of BSA and a synthetic peptide corresponding to the H3 loop of b12 (66). This neutralizing activity against HIV-1_{MN} and HIV-1_{IIIB} was specific because a conjugate of BSA and an irrelevant peptide did not neutralize virus (66). We present here some additional observations. Whereas neutralization of HIV-1 was found for HIV-1_{MN} and HIV-1_{IIIB} in H9 cells at $\leq 1 \text{ mg}$ of conjugate per ml (IC₇₅ $\approx 0.5 \text{ mg/ml}$ for each [66]), no neutralization at 4 mg of the peptide-BSA conjugate per ml was observed for the primary isolate HIV-1_{JRFL} in a peripheral blood mononuclear cell assay (data not shown). It should be noted that, in our hands, neutralization (IC₉₀) of HIV-1_{JRFL} occurs at $\approx 0.8 \mu\text{g}$ of IgG1 b12 per ml (94), so HIV-1_{JRFL} is as sensitive to neutralization by IgG1 b12 as HIV-1_{IIIB} (IC₉₀ $\approx 0.5 \mu\text{g/ml}$). Thus, although T-cell-line-adapted viruses were neutralized at high concentrations of the b12 H3 peptide conjugate, the primary isolate, HIV-1_{JRFL} was not neutralized by the same conjugate.

We also wished to determine whether a direct interaction between the b12 H3 peptide and recombinant gp120 could be established. By direct ELISA, no specific interaction between the b12 H3 peptide or the b12 H3 peptide-BSA conjugate and gp120 was detected by immobilizing either the peptide or recombinant gp120 (JRFL and IIIB) and then probing with the partnering molecule (data not shown). Similarly, by competition ELISA, high concentrations of b12 H3 peptide (0.5 mg/ml) or b12 H3 peptide-BSA conjugate (4 mg/ml) did not inhibit the ELISA signal generated by Fab b12 against immobilized recombinant gp120 (JRFL and IIIB; data not shown). Thus, it appears that, although the b12 H3 peptide-BSA conjugate neutralizes the T-cell-line-adapted viruses HIV-1_{MN} and HIV-1_{IIIB}, we could not demonstrate a specific interaction between the conjugate and recombinant gp120, at least by direct and competition ELISAs, which may not detect interactions with high micromolar to millimolar dissociation constants. These results may be explained at least in part by differences in the conformation of gp120 as it exists in the trimeric envelope spike, as probed in neutralization assays versus recombinant, plate-immobilized gp120, as probed by ELISA.

DISCUSSION

In the current study, we examined the antigen binding site of b12 with site-directed mutagenesis to create 50 mutations involving 27 different residues in four different CDRs of b12. We

found that the use of crude Fab supernatants was reproducible and efficient, allowing the simultaneous analysis of a large number of Fab mutants against a panel of antigens. This type of mutational analysis can easily be adapted for other Fabs or single-chain Fvs that are amenable to production in *E. coli*, and we are currently using this approach for other antibodies against HIV-1. One potential drawback of the analysis is that if a substitution leads to diminished antigen binding, it is not known whether it is due to a direct effect on affinity for antigen or an effect on Fab stability or folding. However, we targeted residues in the CDR loops, most of which were at least partially exposed to solvent. For buried residues such as V95, N100g, and Y100i, it is possible that Ala substitution might partially unfold these mutants. Nevertheless, of the mutants that were chosen to be purified, W100A, Q100eA, N100gA, Y100hA, A93Y, and Y53G, most gave favorable yields of Fab and produced a single 50-kDa band by SDS-PAGE, suggesting that the Fabs were largely intact and not degraded. Moreover, the purified Fabs generally showed activities against gp120 very similar to those of the crude Fabs.

A very strong correlation was found for binding of the mutants to both gp120_{IIIB} and gp120_{JR-FL}. This correlation is consistent with a common binding mechanism of b12 to both the T-cell-line-adapted and primary isolate gp120 and is perhaps not surprising for an antibody with such broad reactivity to diverse HIV-1 envelopes (11, 54, 79). W100 was found to be important, as its substitution generally decreased binding of b12 to gp120. This result is also consistent with a prior experiment in which a portion of the gene segment encoding the H3 loop of b12, including W100, was randomized and incorporated into a phage display library and the library was affinity selected against gp120. A Trp residue at position 100 was absolutely conserved in all clones selected (5). These data caused us to speculate that perhaps a Trp residue (b12) might be superior to a Phe (CD4) in filling the hydrophobic pocket in gp120 that is important for CD4 receptor engagement (Phe43 is used to fulfill this role in CD4 [39]). However, an F43W mutant of CD4 did not show enhanced binding to gp120 but rather bound more poorly (i.e., the F43W mutant bound to gp120 at $\approx 9\%$ of wild-type CD4 levels, and F43Y bound gp120 at $\approx 40\%$ of wild-type CD4 levels; Raymond Sweet, personal communication). In addition to W100, the three remaining aromatics in the H3 loop of b12 appeared to be important for gp120 recognition; mutation of any of four aromatics (Y98, W100, Y100h, and Y100i) to Ala resulted in $\geq 95\%$ decrease in relative binding to gp120.

By contrast, mutation to Ala of only one of four aromatics (i.e., residue Y², as defined in Fig. 4) in the H3 loop of the poorly neutralizing anti-CD4bs antibody b6 resulted in a $\geq 95\%$ decrease in relative binding to gp120 and then only to one of the two strains, IIIB (Fig. 4). In fact, for the Y²A mutant of Fab b6, a much greater binding differential was found between gp120 strains than for any of the 50 b12 mutants (Fig. 4). This result highlights a striking difference in the way that the aromatics of b12 and b6 are used to bind gp120, at least with respect to their H3 loops, even though the footprints of b6 and b12 on gp120 appear to be relatively similar (55). The H3 loop of an antibody is usually crucial in determining its specificity (82, 89). It may be that W100 (b12) and Y² (b6), each of which is located near the middle of their long H3 loops, probe dif-

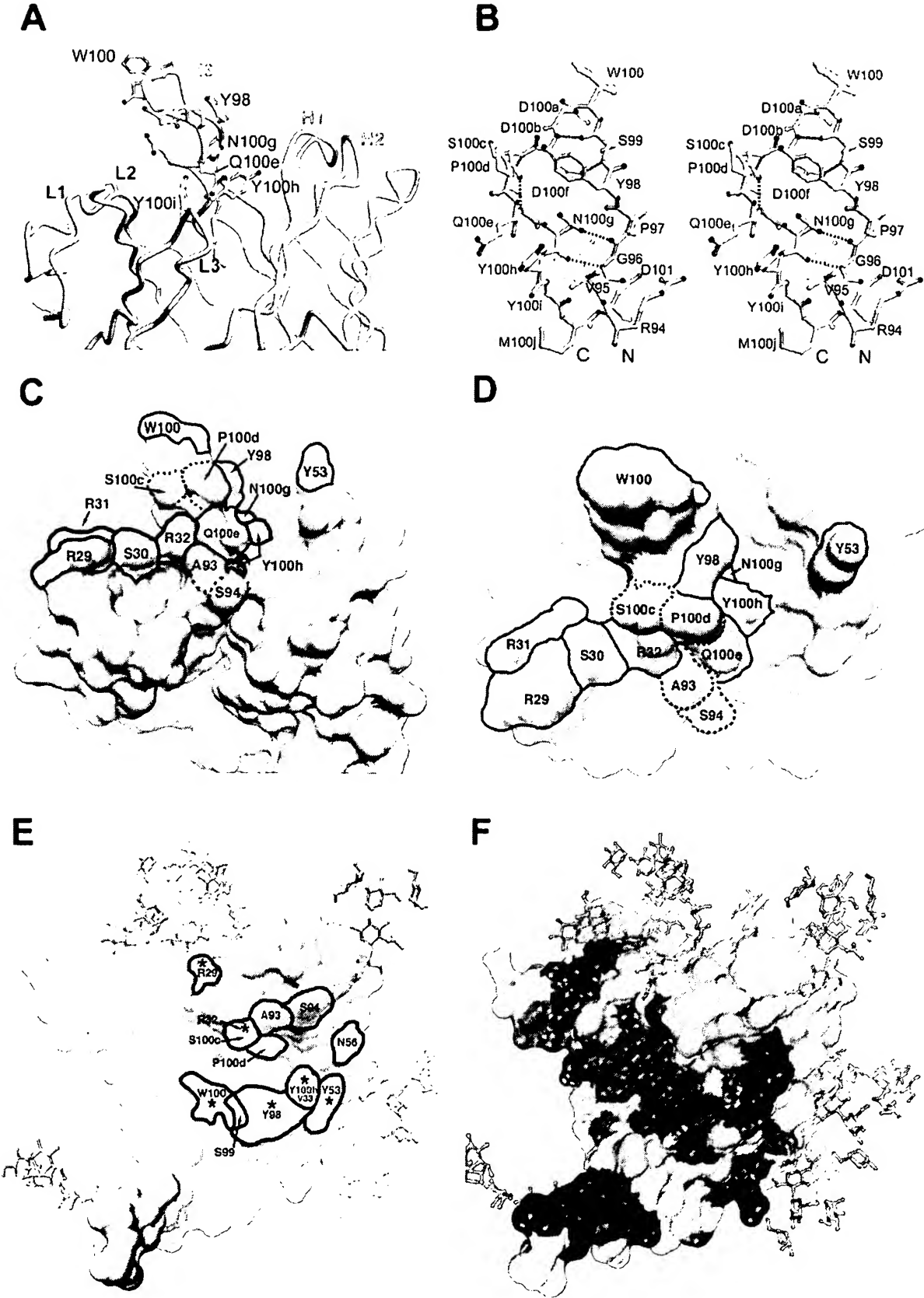
ferent regions within the CD4bs of gp120. Broadly neutralizing molecules such as b12 and CD4 might be able to occupy the conserved hydrophobic pocket of gp120, whereas b6 may not possess this ability. We speculate that the b6 H3 loop lacks rigidity and might lie across the CD4bs of gp120 rather than inserting into it like b12. Given that the Y²A mutation of b6 leads to significant strain preference (Fig. 4) but W100A of b12 does not, the Y² residue could be interacting with or proximal to a region of gp120 that is relatively variable.

Recently, Zhu et al. showed that an engineered molecule (MBri) containing the V1/V2 loop and a portion of the bridging sheet of gp120 (i.e., $\beta 2$, $\beta 3$, $\beta 20$, and $\beta 21$) was able to bind to b12 (91). These authors showed that a V3 loop peptide is able to partially inhibit the binding of b12 to MBri and attributed this effect to a physical interaction between the V1/V2 loop and V3 loop. One might speculate that b12 is able to bind to gp120 in spite of an interaction between variable loops on the native trimer, whereas other anti-CD4bs antibodies, such as b6, cannot. In this vein, we have observed that most anti-CD4bs antibodies but not b12 inhibit the binding of a novel loop-dependent antibody to gp120 (MBZ; Robert Kelleher, Richard Jensen, Aran Labrijn, Meng Wang, Gerald Quinnan, Paul W. H. I. Parren, and Dennis R. Burton, submitted for publication), suggesting that the variable loops affect other anti-CD4bs antibodies in a manner different from how they affect b12.

Further comparative studies, both structural and functional, between b12 and other nonneutralizing anti-CD4bs antibodies like b6 could help elucidate the conformational differences between monomeric gp120 and trimeric gp120 on the HIV-1 envelope spike. In terms of vaccine design, these types of analyses should be extremely helpful to the design of improved gp120 constructs that would elicit b12-like antibodies by maximizing the exposure and immunogenicity of the b12 epitope while limiting the antibody response against overlapping epitopes targeted by b6 and other poorly neutralizing anti-CD4bs antibodies.

A molecular (ribbon) model of the Fab of b12 (Fig. 7A) illustrates the relative orientations of the CDRs and the prominent H3 loop. Figure 7B details the contour of the H3 loop and how the H3 polypeptide extends down on either side from W100 in an extended β -ladder with a distinct twist. The β -ladder is roughly 4 Å in width and contains five hydrogen bonds between strands; most notably, the main-chain carbonyl of G96 hydrogen bonds with the amino group of the side chain of N100g. Our data strongly suggest that this stabilizing hydrogen bond is crucial for the interaction of b12 with both gp120 and B2.1, since mutation of N100g to A, D, Q, H, or Y completely abolishes b12 binding to gp120 and B2.1. Another potentially loop-stabilizing hydrogen bond exists between Q100e and A93, and we found that changing Q100e to Ala, Asn, or Phe resulted in diminished binding to gp120. We note that in a recent and related study by McHugh et al. (47), it was shown by mutational analysis of a single chain Fv fragment corresponding to a b12 variant, 3B3-PE, that gp120 recognition was enhanced ≈ 2 -fold by a Q100eY mutation. Thus, it appears that an aromatic residue at Q100e is compatible with strong gp120 binding in certain contexts.

The last few C-terminal residues of the H3 loop of b12 are encoded by DNA contributed by the joining region, which, in



the case of b12, belongs to the family JH6 (4). The JH6 segment of DNA can potentially contribute up to six consecutive Tyr residues. We considered the possibility that this portion of the H3 loop of b12 may be a part of a more conserved and general structural motif, since a single segment of DNA encodes it as a group. We performed a database search (1) on the motif NYMDV and found only three exact matches: one is an antihapten human heavy chain, VH-37 (accession no. S46393 [25]), and two are highly homologous antibodies against rhesus D blood group antigen (accession no. Y08177 and Y08186; S. M. Miescher, personal communication). Unfortunately, no structure for these antibodies is currently available to compare with that of IgG1 b12.

The strong dependence of b12 on a CDR of the light chain is consistent with a previous chain-shuffling experiment in which the heavy chain of b12 exclusively paired with the same light chain following affinity selection on gp120 (4). Significantly, of the six non-H3 mutations that were found to decrease gp120 binding by $\geq 90\%$, four arose via somatic mutation, and three of these were in L1. [The four substitutions, G53Y(H2), S29R, S31R, and Y32R, (L1) were determined to have arisen via somatic mutation on the basis of sequence alignment (<http://imgt.cnusc.fr:8104/home.html>) with known human germ line genes (see Fig. 1), and their positions in the paratope of b12 can be seen in Fig. 7C and D.] Although germ line antibodies can neutralize some viruses (35), given our results, it is very unlikely that a nonsomatically mutated version of b12 would have any HIV-1-neutralizing activity.

In a prior experiment, the gene segment encoding six residues in L1 (including R29, R31, and R32) was randomized and incorporated into a phage display library, and the library was affinity selected against gp120. Although there was positive selection for Arg residues in the enriched phage pools, the highest-affinity variant differed from b12 at every targeted position but R32, which was absolutely conserved in all the clones sequenced (90). The L1 sequence of the highest-affinity Fab is identical to that of H31L42, its whole-IgG counterpart (37) (Fig. 1). Note, however, that the multiple substitutions in CDR L1 of H31L42 occurred in the background of additional substitutions in H3 and H1, and the effects of most of these substitutions on affinity for gp120 were nonadditive (90).

A footprint of the putative contact residues of b12 on the deglycosylated core of gp120 (39), according to our docking model (66), is shown in Fig. 7E. The b12 residues whose substitution diminished gp120 binding by $\geq 95\%$ relative to the

wild type are indicated by asterisk. A useful guide in orienting the two molecules is the insertion of the "fingers" H3 (W100) and H2 (Y53) of b12 into the CD4 hydrophobic pocket and into a gap between T373 and N386 in gp120, respectively. The diminished gp120 binding of mutants W100A, Y98A, and Y53G are supportive of and supplement the model.

We postulate that W100 and Y53 contribute to the binding energy of b12 to gp120 largely by burying into a hydrophobic pocket and canyon, respectively, on either side of a ridge including S365 and D368 on gp120. Y98 of b12 is also very close to the S365-D368 ridge, which has been shown to be important for b12 binding by mutational analysis (55). Residues R29 and R32 in L1 are in close proximity to residues N276/K282 and N280/A281 in the D-loop of gp120, respectively. A recent mutational analysis of gp120 shows that substitutions N276A, K282A, and N280A slightly enhance, diminish, and have no effect on the binding of b12 to gp120, respectively (55).

From the crystal structure of b12, we calculated that the side chain of R32 was only partially accessible to solvent (Fig. 1), suggesting that the observed effect of the R32Y substitution may be due more to changes in local paratope structure than to direct contact with gp120. By contrast, the side chain of R29 is mostly accessible to solvent, suggesting that this residue might contact gp120. Alternatively, long-range electrostatic effects due to the cluster of basic residues in L1 might play a significant role in the observed effects caused by replacing any one of these b12 residues. N56 of b12 docks in close proximity to K362 of gp120; however, the N56 side chain points away from the hydrophobic canyon into which Y53 is situated, potentially explaining the absence of an effect on gp120 binding of substituting this residue. We would also add the caveat that some Ala substitutions can be energetically neutral in receptor-ligand interactions, despite replacing contact residues, as found for example in the Fab D1.3-hen egg white lysozyme complex (18). Exactly how the b12 residues discussed above spatially relate to residues in gp120 will require determination of a crystal structure of b12 in complex with gp120.

Despite the extreme differences between gp120 (≈ 120 kDa, heavily glycosylated protein) and the B2.1 peptide (dimer ≈ 4.3 kDa, nonglycosylated peptide), there are many similarities in the effects of b12 mutations on its binding to these antigens: 36 mutations had qualitatively similar effects, whereas 14 had different effects. A recently solved crystal structure of Fab b12 in complex with the B2.1 peptide should help in identifying which of these residues represent differential contacts for B2.1

FIG. 7. Molecular features of the paratope of b12. (A) Tube diagram (36) of the combining site of Fab b12, showing the position of the protruding H3 loop relative to the other CDRs. Residues in H3 for which replacement by Ala resulted in a $\geq 95\%$ decrease in apparent affinity to gp120 relative to wild-type (w.t.) b12 are labeled. (B) Stereo diagram of a ball-and-stick representation (24, 36) of the H3 loop of b12. The key residues that were labeled in A are labeled in red. (C and D) Molecular surface rendering (64, 65) of the b12 paratope (C, side view; D, top view). The light chain is colored pink, and the heavy chain is colored yellow. Residues that upon substitution caused a $\geq 95\%$ decrease in apparent affinity to gp120 relative to wild-type b12 are indicated (solid outline). Note that residue Y100i is buried. Residues S100c, P100d, A93, and S94 (dotted outline) are shown for facile comparison with panel E. The b12 structure is taken from Sapphire et al. (66). (E) Crystal structure of the gp120 core (39) with the residues of b12 that are predicted from the docking model (66) to be in close proximity to the outlined region on gp120. Thus, the labeled residues are those of b12, not gp120. Putative footprints in pink and yellow are from light- and heavy-chain residues, respectively. Asterisks (*) indicate the predicted contact residues that were also found to be critical for gp120 recognition by mutational analysis in this study, as defined in panels C and D. Note: a crystal structure of b12 in complex with core gp120 is as yet unavailable. (F) Sequence conservation map of core gp120 as defined by Kwong et al. (39). Residues in blue are conserved among all primate retroviruses, residues in green are conserved among all HIV-1 isolates, residues in yellow are moderately conserved among all HIV-1 isolates, and residues in grey are variable. Carbohydrate has been modeled onto the core structures (39) of gp120.

and gp120 (E. O. Saphire, M. Montero, A. Menendez, M. B. Irving, M. B. Zwick, P. W. H. I. Parren, D. R. Burton, J. K. Scott, and I. A. Wilson, submitted for publication). The D100fE mutation, in particular, is interesting because it is conservative yet it severely impairs b12 binding to B2.1, whereas gp120 binding is almost unchanged. In addition, mutations D100aA, D100bA, 3D3A, and 3D2N all enhance b12 binding to B2.1 but reduce binding to gp120, suggesting that the "acidic patch" on the H3 loop (66) is more favorable for binding to gp120 than to B2.1. It is also noteworthy that binding of the W100 mutants to the B2.1 peptide was the same as wild-type b12, which may be expected since B2.1 does not have a deep hydrophobic cavity like gp120.

IgG1 b12 is one of a very few antibodies that exhibit potent cross-isolate anti-HIV-1 neutralizing activity (7, 8, 11, 21, 37, 79). It is therefore instructive to gather structural and functional information with respect to the neutralizing activity of IgG1 b12 in the hope of learning how to reproducibly elicit b12-like antibodies. From the antibody perspective, a question that surfaces in regard to this goal is how close to b12 does an antibody need to be in order to have the same ability to neutralize HIV-1. Two possibilities may exist: b12 is a unique specificity that cannot be reproduced with antibodies with low sequence homology to b12, or the specificity of b12 may be reproduced by a wide spectrum of nonhomologous antibodies.

Obviously, the former possibility would present a much greater challenge to template-driven vaccine design in that only anti-CD4bs antibodies that use the same germ line genes as b12 and have the correct critical residues in the CDRs would be broadly HIV-1 neutralizing. Some of these crucial residues are encoded by non-germ line DNA sequences. However, one of the very hallmarks of the humoral immune response is its ability to devise novel solutions to biomolecular recognition with molecules that have low sequence homology (i.e., the variable regions of antibodies) yet can still recognize the same epitope. Such plasticity in antigen recognition has been observed in other ligand-receptor systems (84). Clearly, additional broadly neutralizing anti-CD4bs antibodies are sorely needed in order to determine whether or not the key molecular features of b12, such as the long and rigid CDR H3 finger, the positively charged shelf-like structure in L1, and the high level of somatic mutation in b12, are required of other anti-CD4bs antibodies in order to be as broadly neutralizing against HIV-1.

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Retroviridae

Definition: Family of RNA viruses that infects birds and mammals and encodes the enzyme reverse transcriptase. The family contains seven genera: HTLV-BLV VIRUSES; LENTIVIRUS; RETROVIRUSES TYPE B, MAMMALIAN; RETROVIRUSES TYPE C, AVIAN; RETROVIRUSES TYPE C, MAMMALIAN; RETROVIRUSES TYPE D; and SPUMAVIRUS. A key feature of retrovirus biology is the synthesis of a DNA copy of the genome which is integrated into cellular DNA. After integration it is sometimes not expressed but maintained in a latent state (PROVIRUSES).

Synonym(s): Leukemogenic Viruses / Leukoviruses / Oncornaviruses / Oncovirinae /

Narrow term(s):

HTLV-BLV Viruses	Lentivirus	Retroviruses Type B, Mammalian
Retroviruses Type C, Avian	Retroviruses Type C, Mammalian	Retroviruses, Simian
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






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
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
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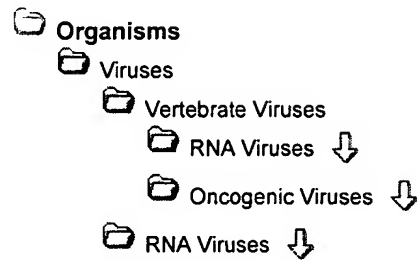
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Binding of HIV-1 gp120 to the nicotinic receptor

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We previously described a significant sequence homology between HIV-1 gp120 and the functional sites responsible for the specific binding of snake curare-mimetic neurotoxins and rabies virus glycoprotein to the nicotinic acetylcholine receptor. Here we report findings about the existence of a mechanism of functional molecular mimicry which could enable the binding of HIV-1 gp120 to nicotinic acetylcholine receptors in muscle cells and neurons.

Acetylcholine receptor; HIV-1; gp120; α -Bungarotoxin; Mimicry

1. INTRODUCTION

In a previous paper [1] we reported a significant homology between the sequence, 164–174 [2], of HIV-1 gp120 and the putative active sites of snake curare-mimetic neurotoxins and rabies virus (RV) glycoprotein, which specifically bind to the nicotinic acetylcholine receptor (AChR). Curare-mimetic neurotoxins from Elapid snakes bind with high affinity to AChR and competitively block acetylcholine-induced membrane depolarization [3]. On the other hand the rabies virus binds to the muscle nicotinic receptor and this binding is inhibited by snake neurotoxins [4]. We consider the homology of gp120 with snake neurotoxins and RV glycoprotein to be of potential importance for HIV-1 infectivity in that it is centered around a region comprising highly conserved snake neurotoxin residues probably involved in receptor binding; moreover, in rabies virus glycoprotein, the same sequence corresponds to the site of rabies virus binding to AChR [5,6].

We proposed that nicotinic acetylcholine receptors can function as HIV-1 receptors in muscle cells and neurones, by virtue of mimicry of receptor-specific active sites of ligands by HIV-1 gp120. A similar mechanism is already suspected for rabies virus binding to muscle cells [6].

We found that recombinant gp120 from HIV-1 strain IIIB is able to inhibit the binding of the snake neurotoxin, α -bungarotoxin (α -Bgt), to the nicotinic acetylcholine receptor in the human rhabdomyosarcoma cell line, TE671. A 14-amino acid synthetic peptide (HG165-178: Asn-Ile-Ser-Thr-Ser-Ile-Arg-Gly-Lys-Val-Gln-Lys-Glu-Tyr), reproducing the sequence 165–

178 of gp120, homologous to snake neurotoxins and rabies virus glycoprotein, is also able, once conjugated to keyhole limpet hemocyanin (KLH), to inhibit the binding of α -Bgt to TE671 nicotinic acetylcholine receptor. Further, immunization of mice with the same gp120-derived peptide gave rise to antibodies efficiently cross-reacting with rabies virus glycoprotein and α -Bgt.

2. MATERIALS AND METHODS

2.1. Cell culture

The human cell line, TE671, was obtained from the American Type Culture Collection. Cells were grown to confluence at 37°C in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal calf serum.

2.2. Iodination of α -bungarotoxin

α -Bgt was obtained from Sigma (St. Louis, MO, USA) and labelled with 125 I as described [7]; specific activity was $2\text{--}3 \times 10^{17}$ cpm/mol.

2.3. [125 I] α -Bgt binding to TE671 cells and inhibition by HIV-1 gp120

For binding experiments, cells were harvested mechanically with a rubber policeman and centrifuged at $450 \times g$ for 15 min; pellets were resuspended in phosphate buffered saline (PBS), pH 7.5, to a density of 10^7 cells/ml; 10^6 cells were incubated with 50 μ l of serial dilutions of HIV-1 gp120 (IIIB strain, Neosystem Laboratoire, Strasbourg, France) for 3 h under gentle stirring. 50 μ l of [125 I] α -Bgt (10^3 cpm) were then added and the cells incubated for a further 45 min. Binding was stopped by the addition of 1 ml ice-cold PBS containing 1 mg/ml BSA and samples were then centrifuged at $450 \times g$ for 15 min at 4°C. The cell pellets were washed twice as above and counted in a γ -counter (Minimaxi 500, Packard Instruments Co., Downers Grove, IL). Maximum binding was obtained by replacing inhibitors with assay buffer. Non-specific binding was determined in the presence of 7.5×10^{-6} M unlabelled α -Bgt. [125 I] α -Bgt binding in the presence of 1 mM nicotine and of 25 mM acetylcholine/0.25 mM neostigmine was also measured for additional controls.

2.4. Peptide synthesis

Solid phase synthesis was carried out with a model 430A automatic synthesizer (Applied Biosystems, Foster City, CA) employing F-moc chemistry. The peptide sequence was checked by a gas-phase microse-

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quencer (Model 470A, Applied Biosystem). HG165-178 was conjugated with KLH by glutaraldehyde, about 300 mol of peptide were bound per mol of KLH. The conjugated peptide (cHG) was used for inhibition experiments and for immunization of mice.

2.5. Inhibition of [125 I] α -Bgt binding to TE671 AChR by HG165-178 KLH conjugated peptide

5×10^5 cells in 100 μ l of PBS were incubated with 50 μ l of serial dilutions of peptides for 2 h. [125 I] α -Bgt (10^5 cpm) were then added and incubated for 45 min. The cells were washed and radioactivity counted as described above. Maximum binding was determined by replacing inhibitors with assay buffer. KLH and an uncorrelated 14-amino acid peptide conjugated to KLH (S1) were used under the same conditions to check non-specific inhibition.

2.6. Antibodies

Balb/c mice were injected intraperitoneally with 250 μ g of HG165-178 KLH-conjugated peptide in complete Freund's adjuvant (CFA) (day 1). The mice were boosted as above using incomplete Freund's adjuvant at days 15 and 36. At day 57, the mice were injected intravenously with 100 μ g conjugate in saline. After 3 days serum from immunized animals was collected for the experiments (polyclonal antibodies).

2.7. ELISA

Anti-HG165-178 mouse antiserum was tested on three different antigens. 96-well EIA plates were coated with HIV-1 gp120, RV glycoprotein or α -Bgt in 50 mM ammonium carbonate buffer, pH 9.5, for 18 h at 4°C. The plates were then washed and quenched with 3% bovine serum albumin, washed again and incubated with serial dilutions of antiserum for 3 h at 37°C. Binding was detected by horseradish peroxidase-conjugated anti-mouse IgG.

3. RESULTS AND DISCUSSION

A significant homology is present between the sequence, 164–174, of HIV-1 gp120 and the active sites responsible for the binding of snake neurotoxins and rabies virus glycoprotein to muscle AChR (Fig. 1).

The human rhabdomyosarcoma cell line, TE671, is known to express a muscle-like nicotinic receptor [8]. [125 I] α -Bgt binding to the nicotinic receptor in the TE671 cell line was measured on intact cells. Non-specific binding was checked in the presence of a high excess of unlabelled α -Bgt. No binding of [125 I] α -Bgt was detected on hepatoma PLC/PRF/5 (data not shown), a different human cell line. Gp120 from the HIV-1 strain IIIB was found to inhibit the binding of α -Bgt to TE671: 30% of maximum binding (B_0) was measured in the presence of 2×10^{-7} M gp120 (Fig. 2). Acetylcholine and nicotine were used under the same conditions to specifically inhibit the binding, as already described for α -Bgt binding to torpedo AChR [7,9]. HIV-1 gp120 inhibition of α -Bgt binding indicates a 'functional equivalence' between these proteins for nicotinic

C-D-I-F-T-N-S-R-G-K-R	RV glycoprotein (residues 189-199)
F-N-I-S-T-S-I-R-G-K-V	HIV-1 gp120 (residues 164-174)
C-D-A-F-C-S-I-R-G-K-R	α -cobratoxin (residues 30-40)
C-D-A-F-C-S-S-R-G-K-V	α -bungarotoxin (residues 30-40)

Fig. 1. Sequence homology of HIV-1 gp120 with rabies virus glycoprotein and snake venom neurotoxins.

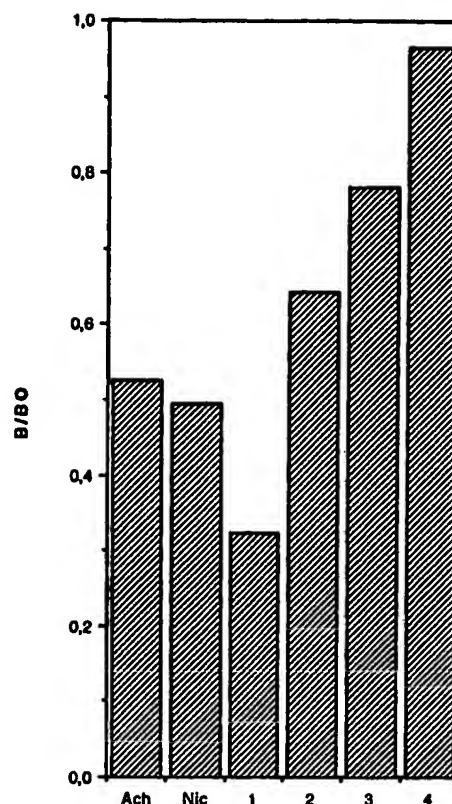


Fig. 2. [125 I] α -Bgt binding to TE671 AChR in the presence of (Ach) 2.5×10^{-2} M acetylcholine/ 2.5×10^{-4} M neostigmine; (Nic) 10^{-3} M nicotine; (1) 2×10^{-7} M gp120; (2) 10^{-7} M gp120; (3) 5×10^{-8} M gp120; (4) 2.5×10^{-8} M gp120. Each point is the mean of duplicate determinations after subtraction of non-specifically bound radioactivity (B_n). Maximum binding (see text).

receptor binding. To investigate whether the sequence of HIV-1 gp120, homologous to snake neurotoxins and rabies virus glycoprotein, might be involved in the binding of gp120 to AChR, we synthesized a 14-amino acid peptide (HG165-78: Asn-Ile-Ser-Thr-Ser-Ile-Arg-Gly-Lys-Val-Gln-Lys-Glu-Tyr), reproducing the sequence 165–178 of gp120. Once conjugated to KLH this peptide inhibits [125 I] α -Bgt binding to intact TE671 cells (Fig. 3). About 40% of maximum binding was obtained in the presence of 1.4×10^{-7} M conjugated peptide, but we could not measure any significant inhibition with the free peptide.

The effect of the protein carrier is not surprising. It has been reported in other cases and attributed to the stabilization of the peptide active conformation [10]. In our case the further ability of gp120 to inhibit the binding of α -Bgt to the nicotinic receptor in TE671 seems to indicate that the HG165-178 active conformation is similar to the one this sequence assumes in the native protein. Moreover immunization of mice with KLH-conjugated HG165-178 gave rise to an antiserum which bound to gp120 and also recognized rabies virus glycoprotein and α -Bgt in ELISA (Fig. 4), confirming

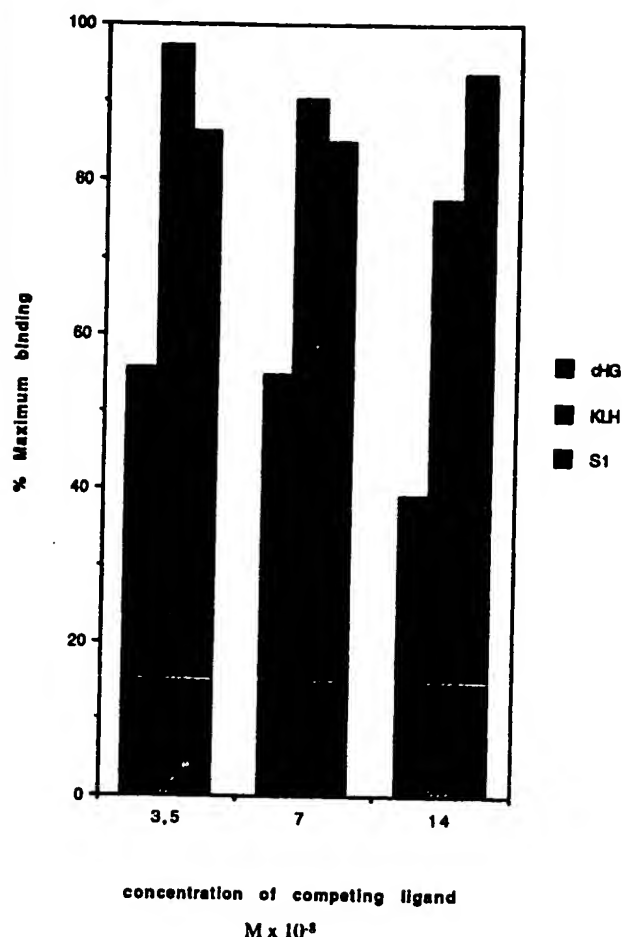


Fig. 3. [¹²⁵I]- α -Bgt binding to TE671 AChR in the presence of different concentrations of the following competing ligands: KLH-conjugated HG165-178 (cHG), KLH, and an uncorrelated 14-amino acid KLH-conjugated peptide (S1).

the possibility of a structural similarity between the regions of these proteins having remarkable sequence homology. In the light of our results we cannot exclude the possibility that inhibition of α -Bgt binding to TE671 cells by HIV-1 gp120 is due, at least in part, to receptor down-regulation following gp120 binding.

The existence of an HIV-1 receptor alternative to CD4 in neurones and muscle cells is strongly suggested by evidence of the ability of HIV-1 to infect CD4-negative muscle and neural cells [11,12] and the lack of inhibition by soluble CD4 of HIV-1 infection of muscle and neuronal cell lines [13]. Galactosyl ceramide has been reported to specifically bind HIV-1 gp120, and has been proposed as an essential component of HIV-1 receptors in neural cell lines and brain cells expressing this or a related lipid [14,15]. Our evidence of the binding of gp120 to TE671 nicotinic receptors helps to explain HIV-1 infection of muscle cell lines, and suggests that nicotinic receptors may also bind HIV-1 gp120 in neural cells. At least two populations of nicotinic receptors are

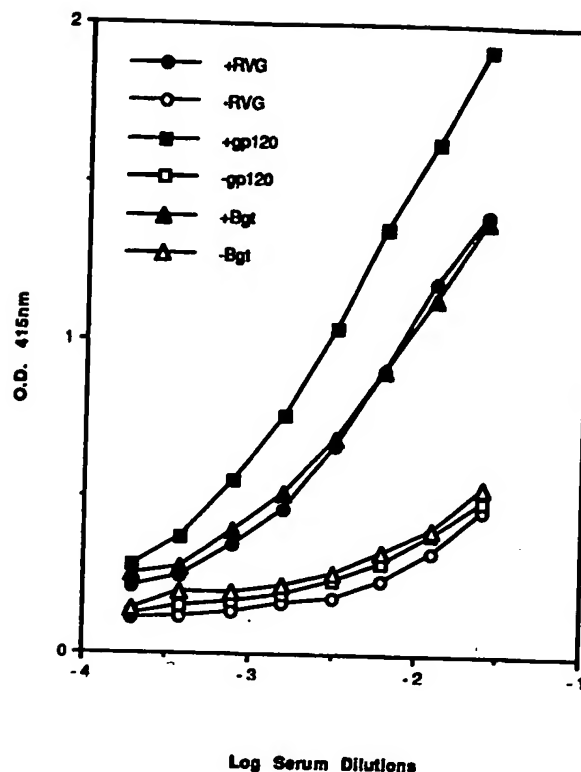


Fig. 4. Anti-KLH-conjugated HG165-178 mouse polyclonal antibodies tested in ELISA on RVG (+RVG), gp120 (+gp120) and α -Bgt (+Bgt), compared to equivalent dilution of normal mouse serum (-RVG), (-gp120) and (-Bgt).

expressed in the nervous system [16], one of which is labelled by α -Bgt. Moreover some of the cell lines, such as RD, TE671 and IMR32, in which a CD4-independent infection by HIV-1 has been proposed, are known to express α -Bgt binding nicotinic receptors [8,17].

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Sequence homology between HIV gp120, rabies virus glycoprotein, and snake venom neurotoxins

Is the nicotinic acetylcholine receptor an HIV receptor?

Brief Report

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Accepted June 14, 1990

Summary. We have found a striking homology between the sequence 164–174 of HIV-1 gp120 and the sequence 30–40 of snake venom neurotoxins; this sequence homology is very similar to that existing between the same region of snake venom neurotoxins and rabies virus glycoprotein.

*

The nicotinic acetylcholine receptor has already been proposed as a host cell receptor for rabies virus during the infection of muscular and neuronal cells [11]. The first indication was discovered by Lentz and co-workers: they found a remarkable sequence homology between a region of the rabies virus glycoprotein and the putative functional site of snake venom curare-mimetic neurotoxins [12], which suggested a functional convergence between these different proteins. Snake venom neurotoxins are selective inhibitors of nicotinic acetylcholine receptor which compete with acetylcholine for binding to the physiologic ligand's binding sites located on receptor α -subunits [5].

Neurotoxins from *Elapidae* and *Hydrophidae* venomous snakes are small polypeptides (7,000–8,000 molecular weight); they are divided into two groups, short (60–62 residues) and long neurotoxins (71–74 residues) [reviewed in 5]; more than 60 different neurotoxins have been sequenced and comparison of their primary structures was made, accompanied by aminoacid chemical modification and studies on the three dimensional conformation: these studies provided useful information about the structure–function relations of this protein family. Snake neurotoxins share quite a constant three-dimensional structure composed of a central core and three loops: loop 2 (the toxic loop) is composed

of highly conserved residues, mainly comprised in the 30–45 sequence (numbering according to [17]), some of which are essential for maintaining functional properties [4].

Since the homology between rabies virus glycoprotein and snake neurotoxins was first described, purified rabies virus glycoprotein was demonstrated to be able to compete with the potent neurotoxin of the snake *Bungarus multicinctus* α -bungarotoxin for the binding to the acetylcholine receptor [1]; moreover anti-peptide monoclonal antibodies, directed to the glycoprotein sequence 190–203 where the homology with snake neurotoxins was most evident, efficiently inhibit the binding of both rabies virus glycoprotein and α -bungarotoxin to acetylcholine receptor [1, 15], thus confirming the existence of a correlation between the snake neurotoxins functional site and a region of rabies virus glycoprotein which appears to be involved in virus binding to the receptor.

While studying the correlation between rabies virus glycoprotein and snake neurotoxins we found that a similar homology with neurotoxins toxic loop is also present in the sequence 164–174 of HIV-1 gp120 (numbering according to [18]); comparison of HIV gp120 with α -cobratoxin from the snake *Naja naja siamensis* shows a stretch of 5 identical residues (Fig. 1) comprising highly conserved residues among the neurotoxins family as well as the invariant R₃₇, G₃₈, and K₃₉ that are most probably involved in receptor binding (Fig. 2). Interestingly, a good homology is also evident with k-bungarotoxin, reported to be a selective marker of neuronal nicotinic receptors [7] (Fig. 1).

This sequence homology may have considerable significance in view of the reported ability of HIV to infect CD4 negative cells in culture [8] and the recently described lack of inhibition by soluble CD4 [3] and anti-CD4 antibodies [19] of HIV binding to muscular and neuronal cells.

In the last two cases, on the basis of their results, the authors conclude that HIV might infect neuronal and muscular cells in a way that is not mediated by CD4. The rhabdomyosarcoma cell line (TE 671) used in the inhibition experiments is known to express a muscular acetylcholine receptor [16]; moreover different members of the nicotinic receptors gene family are expressed in different regions of the mammalian central nervous system [6].

1. R D - - - H R G T I	<i>Naja nigricollis</i> Toxin α (Tx α) (30–40)
2. S D - - - H R G T I	<i>Dendroaspis viridis</i> Toxin 4.11.3 (30–40)
3. C D A F C S S R G K V	α -Bungarotoxin (α -Bgt) (30–40)
4. C D I F T N S R G K R	Rabies virus glycoprotein (189–199)
5. F N I S T S I R G K V	HIV gp120 (164–174)
6. C D A F C S I R G K R	α -Cobratoxin (α -Cbt) (30–40)
7. C D K F C S I R G P V	k-Bungarotoxin (k-Bgt) (30–40)

Fig. 1. Comparison of aminoacid sequence of 5 HIV gp120 (residues from 164 to 174) and 4 rabies virus glycoprotein (residues from 189 to 199) with 1, 2 short and 3, 6, 7 long neurotoxins (residues from 30 to 40)

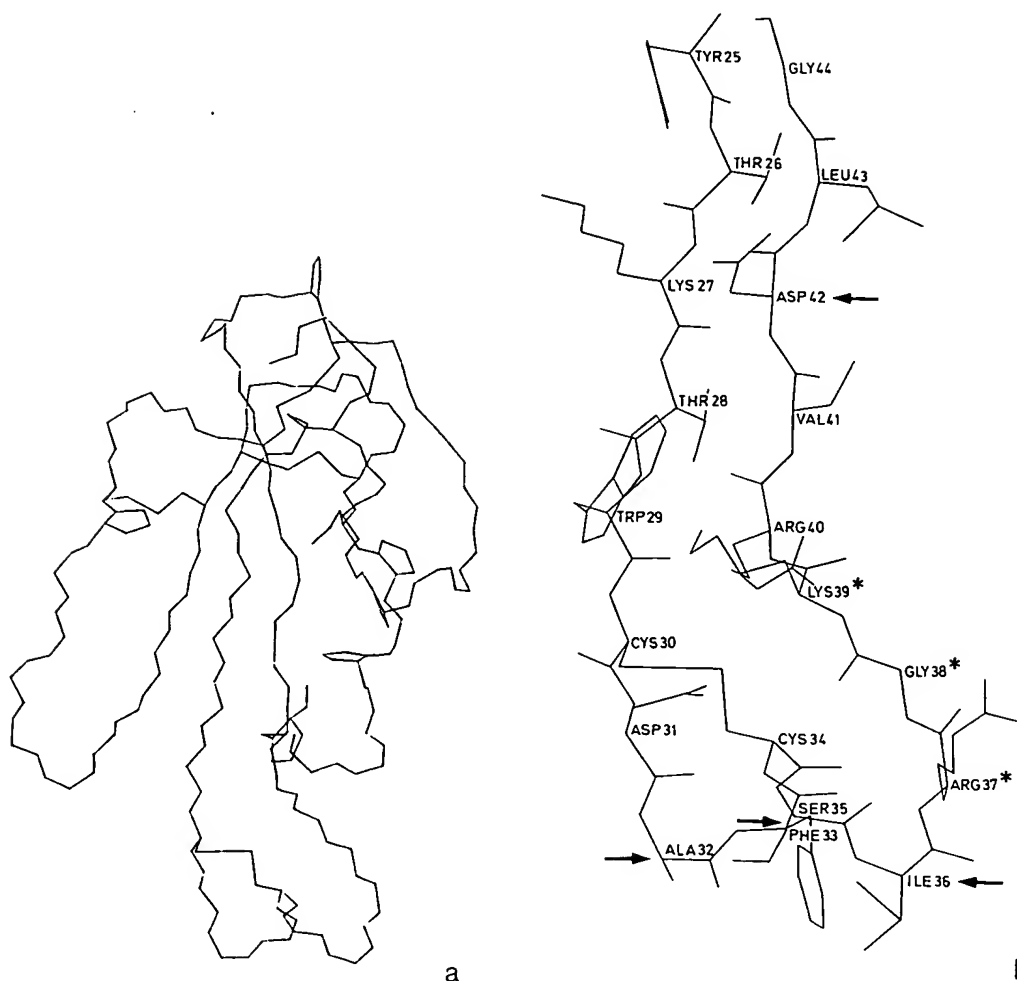


Fig. 2. **a** Computer graphic representation of the main chain folding of the long neurotoxin α -cobratoxin at 0.28 nm resolution. The toxic loop is protruding at the bottom in this representation. **b** Computer graphic representation of the toxic loop of α -cobratoxin. All the residues comprised in this loop are highly conserved among different neurotoxins except for those indicated by arrows; invariant residues are indicated by asterisks

HIV infection of muscular and neuronal cells in vitro might be mediated by gp120 binding to nicotinic receptors present in these cell lines; the possibility that the same mechanism may be the basis of HIV neurotropism should be considered.

It is known that neurologic dysfunctions occur in at least 60% of AIDS patients [10]; subacute encephalitis (AIDS encephalopathy or dementia complex) is the most common neurologic problem which seems to be specifically induced by HIV [10, 14]. Moreover the virus has been isolated in the brain, peripheral nerves, and cerebrospinal fluid of AIDS patients with subacute encephalitis [9, 13].

A sequence homology between a virus and a cellular receptor ligand, such as that observed between rabies virus or HIV and snake neurotoxins, may also be important in view of the possibility that antibodies to the cell receptor could be produced via an anti-idiotypic immune response following an infection with the virus or, through immunization with viral proteins containing the ligand-mimicking sequence. In the case of rabies virus, mice immunized with purified rabies virus glycoprotein produced auto-antibodies directed to the acetylcholine receptor [2], lost weight and died in a short time.

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The Neurotoxin-Like Sequence of Human Immunodeficiency Virus GP120: A Comparison of Sequence Data from Patients With and Without Neurological Symptoms

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Key words: HIV-1, *env*, gp120, neurotoxin, PCR, sequence, neurotropism, receptor

Abstract

A region of the human immunodeficiency virus type 1 (HIV-1) envelope glycoprotein gp120 has been claimed previously to be homologous to parts of snake venom neurotoxins and rabies virus glycoprotein ("the neurotoxic loop"). We have determined DNA sequences directly from a polymerase chain reaction amplified fragment corresponding to this region of HIV-1 gp120 and have translated these to protein sequences. This was performed with the prototype HIV_{SF2} isolate and several Swedish HIV-1 strains, which were precultivated from blood cells or cerebrospinal fluid (CSF) or were directly obtained from CSF cells of patients with and without neurological symptoms. The results show that there are sequence similarities between a short segment of gp120 of clinical HIV-1 strains and the neurotoxic loop. The strains of patients with neurological symptoms did not, however, show a genetic shift of their sequences towards a greater similarity to the sequences of snake venom neurotoxins and rabies virus glycoprotein as compared to the strains of asymptomatic individuals.

Introduction

Human immunodeficiency virus type 1 (HIV-1) commonly infects the central nervous system (CNS) of patients in all stages of infection (1-3). Furthermore,

The aim of this investigation was to study whether a genetic similarity can be found between HIV-1 strains from patients with and without advanced CNS dysfunction and the neurotoxic loop of snake venoms and rabies virus glycoprotein.

Materials and Methods

Subjects

Subjects

The HIV-1 isolates were recovered from peripheral blood mononuclear cells (PBMC) and cerebrospinal fluid (CSF) of six HIV-1 infected patients (no. 1-6; Table 1). The isolates, which had been kept frozen at -70°C for 1-3 years, were thawed, inoculated on cultures of PBMC of healthy blood donors, and maintained as described earlier (2). The patients were clinically examined for neurological symptoms and by ultra low-field magnetic resonance imaging (MRI) of the brain (6). DNA was obtained directly from the CSF cells of two more patients (no. 7 and 8). Analysis was also performed on the DNA of three HIV_{SF2}-infected HuT-78 cultures.

PCR

PCR

Using the DNA of cultured HIV isolates, PCR was performed in the conventional manner using one set of primer pairs, BJTOX2 and BJTOX3 (Table 2). When amplification was carried out using the DNA of CSF cells, the PCR was performed in a nested configuration with a new upstream outer primer, BJTOX1, and BJTOX3 as the inner primer (run with BJTOX2; Table 2). Primers were located in conserved segments of the HIV-1 "neurotoxin-like" genomic region of gp120 (10), corresponding to nucleotide position 319-760 of the HIV_{SF2} *env* reading frame. The 40 cycle configuration of the conventional PCR was 95°C, 30 sec; 55°C, 30 sec; and 72°C, 60 sec, except cycle 1, where denaturation was for 5 min, and cycle 40, where elongation was for 5 min. The same cycle configuration was used in the nested PCR, but the first round of amplification with the outer

Table 1. Characteristics of the patients

Patient no.	Stage of infection
1	AIDS
2	AIDS
3	AIDS
4	PGL
5	Asymptomatic
6	PGL
7	ARC
8	AIDS

PBMC = peripheral blood mononuclear cells; Lymphadenopathy = enlarged lymphadenopathy; ARC = AIDS-related complex; *Determined by magnetic resonance imaging.

primer pair was for 30 cycles. 25 μ l (total volume) of fresh reaction mixture was used using the inner (BJTOX) controls were included.

A check for specificity was performed by agarose gel electrophoresis. The appearance of fragments of the expected size was considered specific.

DNA sequencing

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Table 2. Neurotoxin-like env

	Se
Outer	
BJTOX1	T
BJTOX2	T
Inner	
BJTOX3 ^a	A

^aRun as a nested primer with

ons occur frequently, especially detected in CD4⁺ macrophages. The virus can also establish a latent infection (7). It has, however, been shown to bind to other receptors, e.g., the acetylcholine (ACh) receptor has been shown to bind (10). Thus, a region of the glycoprotein is suggested to be homologous to the glycoprotein of the neurotoxic virus (10,11). A genetic similarity can be found between the glycoproteins of the AIDS virus and rabies virus glycoprotein.

Peripheral blood mononuclear cells from 11 infected patients (no. 1–6; 7 and 8) at -70°C for 1–3 years, were used as blood donors, and maintained at -70°C until examined for neurological symptoms. Magnetic resonance imaging (MRI) of the brain was performed in two more patients (no. 7 and 8) and three HIV_{SF2}-infected HuT-78 cells.

PCR was performed in the conventional manner using the outer primer BJTOX3 (Table 2). When CSF cells, the PCR was performed using the inner primer BJTOX1, BJTOX2, and BJTOX3 (Table 2). Primers were designed to amplify a "neurotoxin-like" genomic region of 319–760 of the HIV_{SF2} *env* gene. The conventional PCR was 95°C , 30 s; 55°C , 1 min; 72°C , 1 min, where denaturation was for 1 min. The same cycle configuration was used for amplification with the outer

Table 1. Characteristics of the patients

Patient no.	Stage of infection	Source of the virus	Neurological symptoms	White matter changes ^a
1	AIDS	PBMC culture	+	+
2	AIDS	PBMC culture	+	+
3	AIDS	PBMC culture	+	+
4	PGL	CSF culture	–	–
5	Asymptomatic	PBMC culture	–	–
6	PGL	CSF culture	–	–
7	ARC	PBMC culture	–	–
8	AIDS	CSF cells	+	+

PBMC = peripheral blood mononuclear cells; CSF = cerebrospinal fluid; PGL = persistent generalized lymphadenopathy; ARC = AIDS related complex.

^aDetermined by magnetic resonance imaging of the brain.

primer pair was for 30 cycles. Then 5 μl of the amplified DNA was added to 50 μl (total volume) of fresh reaction medium, and was run for another 30 cycles using the inner (BJTOX3) and one outer primer (BJTOX2). Frequent negative controls were included.

A check for specificity of the reaction was always performed by running agarose gel electrophoresis (3% NuSieve, FMC Bioprod.) of the amplified product. The appearance of fragments of 441 bp (non-nested) or 401 bp (nested) was considered specific.

DNA sequencing

After the specificity control, the amplified DNA was purified from all the PCR components using a Qiagen PCR purification kit (cat. no. 12313/12315), according to the manufacturer's protocol. The purified DNA was quantified by application

Table 2. Neurotoxin-like *env* primer sequences and their location in the HIV_{SF2} genome

	Sequence (5'-3')	Location
Outer		
BJTOX1	TAATCAGTTTATGGGATCAAAG	6552–6573
BJTOX2	TTCATGTGTACATTGTACTGT	6971–6992
Inner		
BJTOX3 ^a	ATTAACCCCACTCTGTGTTA	6592–6611

^aRun as a nested primer with BJTOX2.

of spots on a plate containing agarose and ethidium bromide. The degree of fluorescence of the spots was compared to a standard dilution series. Double-strand DNA sequencing was carried out directly on the purified DNA using a Multiwell microtiter plate sequencing kit and ^{35}S -thio dATP (400 Ci/mmol) from Amersham (Buckinghamshire, England), essentially according to the company's recommendations, and with the BJTOX3 primer as the sequencing primer. DNA sequence data were stored, edited, and translated to a protein sequence by using the microcomputer program DNASIS of Pharmacia-LKB.

Results

HIV-1 prototype and database sequences

The result obtained from the HIV_{SF2} isolate (FYITTSIRDKI) used in our laboratory concerning the relevant 11 amino acids that correspond to the neurotoxic loop are shown in Fig. 1B. The sequence agrees with the published sequence of

A.			Sequence
Isolate			
HIV-1BRU/HXB2 (164-174/159-169)			F N I S T S I R G K V
HIV-1SF2 (157-167)			F N I T T S I R D K I
HIV-2ROD (165-175)			F N M T G L E R D K K
SIVmm142 (173-183)			F N M T G L K R D K K
B.			Sequence
HIV _{SF2} /SMCL			F Y I T T S I R D K I
C.			Sequence
Patient no	Code	Source	
1	p25931	PBMC	F K V T T N I K D K M
2	p25933	PBMC	F N I T T G I R D K V
3	p25935	PBMC	F N I T T R <u>M</u> R D K V
3	p25936	CSF	F N I T T R <u>I</u> R D K V
4	p25937	PBMC	F N I T T N I R D K V
4	p25938	CSF	F N I T T N I R D K V
5	p26341	PBMC	F N I T T S I R N K V
5	p26342	CSF	F N I T T S I R N K V
6	p26351	PBMC	F N I T T S I <u>K</u> D K V
6	p26352	CSF	F N I T T S I <u>R</u> D K V
7	p2078	CSF	F N V S T S I R D K V
8	p2268	CSF	F Y V T T G I R D K V

Fig. 1. The neurotoxic loop-like amino acid sequences of prototype HIV-1 isolates, HIV-2 and SIV, and patient HIV-1 strains. A: Sequence of two representative HIV-1 isolates, one HIV-2 and one SIV isolate. The data were obtained from the 1990 Los Alamos database (12). B: Sequence of the prototype HIV-1 strain used in this study. The data were derived from PCR-amplified and sequenced *env* DNA. C: Translated sequences of PCR amplified *env* DNA from patients with and without neurological symptoms (see Table I).

this isolate, except for a nonc position (165 in gp120) to a Y N (AAT). Identical results were obtained in cultures that were sequenced.

The neurotoxic loop pattern are summarized in Fig. 2A. Variable amino acids are identical in all of the toxins (4-7 of 8) containing the R₄₀.

The equivalent pattern of sequences from the 1990 Los Alamos database is shown in Fig. 2B. Only the T₁₆₈ is common to all strains containing the amino acid V₁₇₄. In Fig. 2C a comparison of the most commonly used amino acid sequences of HIV-1 gp120 and HIV-2 gp120. The snake toxin pattern. However, only the first 11 amino acids are identical in the HIV-1 gp120 I₁₆₆ and HIV-2 gp120 I₁₆₆.

By inspection of the 1990 Los Alamos database, a sequence motif was found in HIV-2 and simian immunodeficiency virus (SIV) sequences that were identical in the HIV-1 gp120 RDK sequence. The HIV-1 gp120 RDK sequence.

A. Snake toxins: 30-40 (8 sequences)

[E, D] - [G, A, N, I, K] - [E, T] - [G, S]
n = 7 4 7 7

B. "Neurotoxic loop" of HIV-1 gp120: 164-174

[E, Y] - [N, Q, K] - [L, V, M, T] - [I, S]
n = 18 17 12 15

C. Comparison of the most frequently used amino acid sequences of snake toxins, rabies virus glycoprotein and HIV-1 gp120

a. C D G F C S S R G K R (30-40)
b. C D I A T N S R G K R (189-199)
c. F N I T T S I R D K R (164-174)

Fig. 2. The neurotoxic loop-like amino acid sequences of snake toxins, and HIV-1 gp120. Variable amino acids are underlined. The numbers in parentheses are the number of sequences compiled from the database. a: Snake toxins (10 sequences); b: rabies virus glycoprotein (2 strains; 9), and c: HIV-1 gp120 (164-174).

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IRGKV
IRDKI
ERDKK
KRDKK

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IRDKM
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IRDKV
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IRNKV
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SIRDKV
SIRDKV
SIRDKV
GIRDKV

otype HIV-1 isolates, HIV-2 and SIV.
HIV-1 isolates, one HIV-2 and one SIV
abase (12). B: Sequence of the prototype
CR-amplified and sequenced *env* DNA.
patients with and without neurological

this isolate, except for a nonconservative substitution of a N residue in the second position (165 in gp120) to a Y residue, due to an A to T transition in the codon for N (AAT). Identical results were obtained in three HIV_{SF2} isolates from different cultures that were sequenced independently.

The neurotoxic loop pattern obtained with eight different snake toxins (10,11) are summarized in Fig. 2A. Variable amino acids are within brackets. Only three amino acids are identical in all toxins, the D₃₁, R₃₇, and G₃₈. However, a majority of the toxins (4-7 of 8) contain the amino acids C₃₀, G₃₂, F₃₃, C₃₄, S₃₅, S₃₆, K₃₉, and R₄₀.

The equivalent pattern of 19 different HIV-1 gp120 neurotoxic-loop-like sequences from the 1990 Los Alamos database (12) show more variability (Fig. 2B). Only the T₁₆₈ is common to all HIV-1 sequences. A great majority of the HIV-1 strains contain the amino acids F₁₆₄, N₁₆₅, I₁₆₆, T₁₆₇, S₁₆₉, I₁₇₀, R₁₇₁, D₁₇₂, K₁₇₃, and V₁₇₄. In Fig. 2C a comparison is made between sequences compiled from the most commonly used amino acids of snake toxins, rabies virus glycoprotein (11) and HIV-1 gp120. The snake toxins and HIV-1 gp120 both show a S-X-R-X-K pattern. However, only the R-X-K part is shared with the rabies virus glycoprotein sequence. On the other hand, the rabies virus I₁₉₁ and T₁₉₃ show counterparts in the HIV-1 gp120 I₁₆₆ and the conserved T₁₆₈.

By inspection of the 1990 Los Alamos database (12), a neurotoxic-loop-like sequence motif was found at the corresponding positions in *env* of isolates of HIV-2 and simian immunodeficiency virus (SIV; Fig. 1A). Six of the 11 amino acids were identical in the HIV-1_{SF2} and HIV-2_{rod} and SIV_{mm142} isolates, including the RDK sequence. The HIV-2, SIV, as well as all other neurotoxic-loop-like

A. Snake toxins: 30-40 (8 sequences)

[C, D] · D · [G, A, N, I, K] · [E, T] · [G, G] · [S, N, A] · [S, T, I] · R · G · [X, N, E, P] · [B, V]
n= 7 4 7 7 6 4 5 6

B. "Neurotoxic loop" of HIV-1 gp120: 164-174 (19 sequences)

[F, Y] · [N, O, K] · [L, V, M, T] · [I, S] · T · [S, N, V, P, E] · [L, R, L, V] · [B, K, S, G] · [D, G, S, N] · [X, D] · [Y, M, I, T, K, R]
n=18 17 12 15 12 12 15 14 2 18 7

C. Comparison of the most frequently used amino acids of snake toxins, rabies virus glycoprotein and HIV-1 gp120

a. C D G F C S R G K R (30-40)
b. C D I A T N S R G K R (189-199)
c. F N I T T S I R D K R (154-174)

Fig. 2. The neurotoxic loop-like amino acid sequence patterns of snake toxins, rabies virus glycoprotein, and HIV-1 gp120. Variable amino acids are within brackets. The most frequently used amino acids are underlined. The n numbers show the frequency of occurrence of the amino acids. A: Pattern for eight different snake toxins (10,11), from amino acid positions 30 to 40. B: Pattern for 19 different neurotoxic loop-like sequences of HIV-1 gp120 (12), from amino acid positions 164 to 174. C: Comparison of sequences compiled from the most frequently used amino acids of (a) snake toxins, (b) rabies virus glycoprotein (2 strains; 9), and (c) HIV-1 gp120. Identities between the HIV-1 sequence and the other sequences are within boxes.

sequences of 11 amino acids, show a predominant helical or sheet structure of their first half and a coil/turn domain in the second half of the loop, predicted by two different algorithms (13,14).

Sequencing of patient material

When the sequences of the isolates from patients with and without neurological symptoms were compared, there was no obvious change in the total neurotoxic-loop-like sequence towards more similarity to the snake neurotoxins or the rabies virus glycoprotein in the patients with CNS dysfunction (Fig. 1C). Neither was any difference found between patients with and without brain white matter changes detected by MRI. It was not, however, possible to obtain any sequence from the CSF isolates of the two AIDS patients (Fig. 1C, no. 1 and 2) and with the most advanced neurological dysfunction and the largest white matter changes, since these primary isolates did not grow when passaged to the PBMC of healthy blood donors.

Comparison of the sequences from PBMC and CSF isolates of the same patient showed that for two patients (no. 4 and 5) they were identical, and for patient no. 3 there was an M in the PBMC sequence and an I in the CSF sequence (boxed in Fig. 1C). For one additional patient (no. 6) there was a change in the RD(G)K motif, implicated in the neurotoxic loop binding to the ACh receptor. Thus, the PBMC isolate showed a KDK motif, whereas the CSF isolate is changed to a RDK sequence (boxed in Fig. 1C). It should be noted that this was the only change in a total of 20 sequenced and overlapping amino acids from the two sources. This R₃₇ residue in the neurotoxic loop is presumably of special importance for binding to the ACh receptor (10,11). With regard to the RGK motif of the snake toxins and rabies virus glycoprotein, only 2 out of the 19 HIV-1 isolates (HIV_{BRL} and HIV_{HXB2}) of the 1990 Los Alamos database (12) showed this sequence. Twelve of the isolates showed a RDK motif, in accordance with most of our strains (Fig. 1C). One isolate of the Los Alamos database instead had a KNK sequence, a second a KDK, a third a SDK, a fourth a GSD, and a fifth a RSK motif. The two sequenced strains from patient no. 5 showed a unique RNK motif at this position. It should be noted that only one mutation in each step for the S \leftrightarrow G \leftrightarrow D \leftrightarrow N conversion is required in the respective codons AGT \leftrightarrow GGT \leftrightarrow GAT \leftrightarrow AAT used by HIV-1 for these amino acids.

Discussion

The present study reveals the existence of a sequence similarity between HIV-1 gp120 obtained from clinical virus isolates and snake venom neurotoxins. However, no significant difference was found between isolates of patients with and

without CNS dysfunction. Thus, galactocerebroside. Furthermore, based on a glycoprotein, and snake neurotropism could be. Clinical data support the neurotransmitter system in the CNS symptoms are not who also often develop. Furthermore, we have detected by MRI, in HIV described earlier in only determine if the homology was modified in HIV-1 symptoms and brain white matter was found.

If it is hypothesized that of the snake toxins and gp120 sequences, on the probably no, or very little such a structure. Furthermore of the HIV-1 genome with toxic loop sequence. It corresponding to R₃₇) out of the toxins (Fig. 2A) is present that selection towards gp to blood, due to the presence than the CD4 receptor. presumably be more likely to the snake toxins in the

DNA sequencing of quence analysis of a due to selection of HIV ing is performed on virus. Therefore, we also analyzed. Similar results as for the excluded that not only the CSF cells, were adapted to receptor.

It is well known that different populations of between the blood and

t helical or sheet structure of half of the loop, predicted by

with and without neurological change in the total neurotoxic-snake neurotoxins or the rabies infection (Fig. 1C). Neither was it without brain white matter changes possible to obtain any sequence (Fig. 1C, no. 1 and 2) and with the largest white matter changes, assayed to the PBMC of healthy

CSF isolates of the same patient were identical, and for patient no. 1 in the CSF sequence (boxed) there was a change in the RD(G)K to the ACh receptor. Thus, the CSF isolate is changed to a sequence noted that this was the only differing amino acids from the two is presumably of special importance with regard to the RGK motif of only 2 out of the 19 HIV-1 isolates in the database (12) showed this motif, in accordance with most of the database instead had a KNK motif, a fourth a GSD, and a fifth a RSK motif. No. 5 showed a unique RNK motif by mutation in each step for the S codon in the respective codons AGT (HIV-1 for these amino acids).

sequence similarity between HIV-1 and snake venom neurotoxins. However, between isolates of patients with and

without CNS dysfunction. Previous results have indicated that there are alternative sites other than the CD4 receptor through which HIV-1 may enter brain cells. Thus, galactocerebroside has been suggested to play a role in HIV-1 entry (9). Furthermore, based on amino acid homologies between HIV-1 gp120, rabies virus glycoprotein, and snake venom neurotoxins, it has been proposed that HIV-1 neurotropism could be mediated by binding to ACh receptors in neurons (10). Clinical data support the view that disturbances may involve the cholinergic neurotransmitter system in the CNS of HIV-1 infected patients. Thus, extrapyramidal CNS symptoms are not an uncommon finding (15) in HIV-1 infected patients, who also often develop extrapyramidal side effects on antipsychotic drugs (16). Furthermore, we have reported the existence of a brain white matter lesion, detected by MRI, in HIV-1 infected individuals. This type of lesion has been described earlier in only two cases of rabies (6). Therefore, it was of interest to determine if the homologous sequence (corresponding to the neurotoxic loop) was modified in HIV-1 strains from patients presenting with overt neurological symptoms and brain white matter changes such that an even greater homology was found.

If it is hypothesized that there is a true homology between the neurotoxic loops of the snake toxins and rabies virus glycoprotein, on the one hand, and the HIV-1 gp120 sequences, on the other, it must be taken into consideration that there is probably no, or very limited, selective pressure in vivo, on HIV-1 to preserve such a structure. Furthermore, under such conditions the pronounced variability of the HIV-1 genome will also rapidly eradicate any similarities with the neurotoxic loop sequence. It is therefore noticeable that one amino acid (R₁₇₁, corresponding to R₃₇) out of the three conserved D₃₁, R₃₇, and G₃₈ residues in the snake toxins (Fig. 2A) is present in the majority of the HIV-1 strains. It would seem that selection towards greater similarity might be operative in CNS as compared to blood, due to the presence of the nicotinic ACh receptor in higher numbers than the CD4 receptor. Thus, CSF samples from HIV-1 infected patients should presumably be more likely to harbor HIV-1 strains that show stronger similarity to the snake toxins in the neurotoxic loop-like sequence.

DNA sequencing of PCR amplified products is a powerful tool for rapid sequence analysis of a defined, short segment of the HIV genome. Sequence bias, due to selection of HIV strains adapted to culture, can be avoided when sequencing is performed on virus DNA amplified directly from cells of the patients. Therefore, we also analyzed DNA that was obtained directly from CSF cells. Similar results as for the cultured isolates were found. However, it cannot be excluded that not only the virus cultured on PBMC, but also the strains from the CSF cells, were adapted for binding to the CD4 receptor rather than to the ACh receptor.

It is well known that an HIV-1 infected person may harbor several genetically different populations of HIV-1 in the blood (17). Therefore, the genetic divergence between the blood and CSF strains of two patients in our study is not surprising.

The significance of the change in KDK sequence in PBMC to a RDK motif in CSF in one patient is not known. It has, however, been found that single amino acid changes in HIV-1 gp120 can drastically affect receptor binding to the CD4 receptor (18).

It should be noted that the primary virus isolates from the two patients with the most advanced CNS dysfunction did not grow when passaged to new PBMC cultures. It has been discussed earlier whether a change may occur in the tropism of HIV-1 strains in the CNS of patients in the late stages of infection (2,19). Thus, in contrast to the situation for blood cultures, it is not possible to isolate HIV-1 from CSF using PBMC as target cells in a substantial portion of AIDS patients (2). The present results showing an apparent lack of genetic shift in patients with neurological dysfunction towards sequences more homologous to the neurotoxic loop, should, therefore, be interpreted with caution. It cannot be excluded that HIV-1 strains directly obtained from brain cells could exhibit such sequence variation.

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Identification of Structural Elements of a Scorpion α -Neurotoxin Important for Receptor Site Recognition*

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α -Neurotoxins from scorpion venoms constitute the most studied group of modifiers of the voltage-sensitive sodium channels, and yet, their toxic site has not been characterized. We used an efficient bacterial expression system for modifying specific amino acid residues of the highly insecticidal α -neurotoxin Lqh α IT from the scorpion *Leiurus quinquestriatus hebraeus*. Toxin variants modified at tight turns, the C-terminal region, and other structurally related regions were subjected to neuropharmacological and structural analyses. This approach highlighted both aromatic (Tyr¹⁰ and Phe¹⁷) and positively charged (Lys⁸, Arg¹⁸, Lys⁸², and Arg⁸⁴) residues that (i) may interact directly with putative recognition points at the receptor site on the sodium channel; (ii) are important for the spatial arrangement of the toxin polypeptide; and (iii) contribute to the formation of an electrostatic potential that may be involved in biorecognition of the receptor site. The latter was supported by a suppressor mutation (E15A) that restored a detrimental effect caused by a K8D substitution. The feasibility of producing anti-insect scorpion neurotoxins with augmented toxicity was demonstrated by the substitution of the C-terminal arginine with histidine. Altogether, the present study provides for the first time an insight into the putative toxic surface of a scorpion neurotoxin affecting sodium channel gating.

α -Neurotoxins, the most abundant group in Buthidae scorpion venoms, are polypeptides composed of a single chain of 63–65 amino acids, cross-linked by four disulfide bridges, and are responsible for human envenomation (1, 2). They show variability in their apparent toxicity to mammals and insects, in their primary structures, and in their binding features to neuronal membrane preparations (3–6). Among scorpion neurotoxins, the α -group is the most studied and is useful in functional mapping of the sodium channel structure (6; reviewed in Refs. 7–9). Despite the reported structures (10–13), chemical modifications, and immunochemical studies (14–17) of various scorpion α -toxins, the structural elements dictating molecular recognition of their binding site have not been char-

acterized. It was postulated that the molecule surface bearing a cluster of aromatic and hydrophobic residues, termed the “conserved hydrophobic surface,” was associated with the toxic site (10, 13, 18). However, this postulation has not gained experimental support.

Despite the differences in their primary structures and phylogenetic selectivity, scorpion neurotoxins affecting sodium channels are closely related in their spatial arrangements and form a compact globular structure that is kept rigid by four disulfide bridges (1, 19). A genetic approach based on comparative analysis of the variable regions may clarify distinct residues involved in toxin-receptor interactions. However, due to difficulties in producing sufficient amounts of recombinant toxins (20, 21), such an approach has been limited thus far.

Recently, we have established an efficient bacterial expression system of a scorpion α -neurotoxin, Lqh α IT, displaying an exceptionally high insecticidal activity (5, 6, 22). Lqh α IT slows the sodium current inactivation in excitable membranes in a manner characteristic of other scorpion α -toxins (22). Its binding to the receptor site on neuronal membranes is competitively inhibited by the sea anemone toxin ATXII and enhanced by veratridine (6, 23, 24). Lqh α IT binds to a single class of high affinity sites on insect neuronal membranes (K_d = 0.2–0.5 nM and 0.03–0.04 nM in locust and cockroach, respectively (6, 23)) and competes weakly with other α -toxins for binding to rat brain synaptosomes. AaHII, the most potent anti-mammalian scorpion α -neurotoxin, competes for ¹²⁵I-Lqh α IT binding sites on insect sodium channels, suggesting that the two scorpion α -toxins bind to homologous, nonidentical receptor sites on insect and mammalian sodium channels (6). Lqh α IT is toxic to mice at relatively high concentrations as opposed to other scorpion α -neurotoxins such as AaHII (5, 6), yet it appears to recognize an α -toxin binding site on both insect and mammalian sodium channels. Using our functional expression system, we found Lqh α IT very useful for a genetic study (5) and for two-dimensional ¹H NMR studies (25). Here we report the results of modifications introduced at several regions of Lqh α IT that highlight a putative molecular surface-mediating recognition of the receptor site and intoxication.

EXPERIMENTAL PROCEDURES

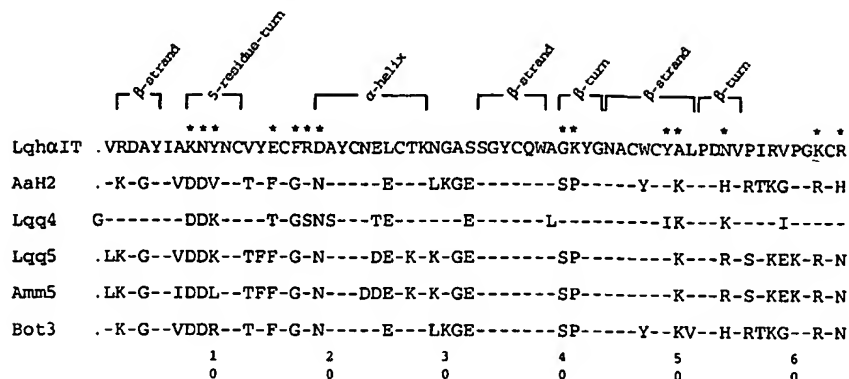
Bacterial and Animal Strains—*Escherichia coli* DH5a cells were used for plasmid constructions. *E. coli* BL21 cells lysogen with DE3 phage derivative bearing the T7 RNA polymerase gene under control of the lac promoter (26) were used for expression. A kanamycin-resistant derivative of pET-11c vector (26) was used for expression (5). *Sarcophaga falcatula* blowfly larvae were bred in the laboratory. Albino ICR mice were purchased from the Levenstein farm (Yokneam, Israel).

Site-directed Mutagenesis, Functional Expression, and Purification of Toxins—Substitutions K8A, K8D, Y10S, Y10W, and K8D/N9D/Y10V were generated by using back-to-back primers and inverse polymerase chain reaction (27) with pBluescript bearing Lqh α IT cDNA (28). Engi-

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FIG. 1. Alignment of the amino acid sequences of several scorpion α -neurotoxins. Residues homologous to those in Lqh α IT are designated by dashes. Dots indicate gaps in the aligned sequences. Modified sites are designated by asterisks (modifications at positions 49, 50, and 54 were described previously (5)), and secondary structure motifs (25) are indicated. AaH2, *Androctonus australis* Hector toxin 2 (AaHII); Lqq4 and Lqq5, *Leiurus quinquestriatus quinquestriatus* toxins 4 and 5, respectively; Amm5, *A. mauretanicus mauretanicus* toxin 5; Bot3, *Buthus occitanus tunetanus* toxin 3 (3).



neering of the 5' and 3' termini of the Lqh α IT cDNA for expression was described previously (5). Substitutions E15A, F17G, R18A, G40S/K41P, and K8D/E15A were performed according to the method of Deng and Nickoloff (29). Expression of the recombinant polypeptides in a non-soluble form, renaturation, and purification of the active toxin were performed as described previously (5). Sequences of the mutated cDNAs were verified prior to expression with Sequenase version II (U. S. Biochemical Corp.). Quantification of the purified recombinant Lqh α IT variants and verification of their composition was performed by amino acid analysis.

Toxicity Assays—Four-day-old blowfly larvae (*S. falcata*; 100 \pm 20 mg body weight) were injected intersegmentally. A positive result was scored when a characteristic paralysis (immobilization and contraction) was observed 5 min after injection. Nine larvae were injected with five concentrations of each toxin in three independent experiments. ED₅₀ values were calculated according to the sampling and estimation method of Reed and Muench (30). Toxicity to mammals was determined by subcutaneous injection to female mice (20 \pm 3 g).

Competition Binding Experiments—Preparation of radiolabeled Lqh α IT was performed according to Gordon and Zlotkin (23). Cockroach (*Periplaneta americana*) synaptosomes (P₂L fraction) were prepared from the central nervous system by established methods (24, 31). The binding assays were performed in the form of equilibrium competition assays using increasing concentrations of the unlabeled toxin in the presence of a constant low concentration of the labeled toxin (30–50 pM) (6). Analyses of binding assays were carried out by using the iterative computer program LIGAND (Elsevier Biosoft). Each experiment was performed at least three times.

Circular Dichroism Measurements and Molecular Modeling—The protein samples used for CD spectrum analyses were in 20 mM phosphate buffer, pH 7. Spectra were measured at 20 $^{\circ}$ C in 0.05-mm path length cuvette from 260 to 178 nm with a JOBIN-YVON (Long-Jumeau, France) UV CD spectrophotometer (Mark VI). Calibration was performed with (+)-10-camphorsulfonic acid. A ratio of 2.2 was found between the positive CD band at 290.5 nm and the negative band at 192.5 nm. Data were collected at 0.5-nm intervals with a scan rate of 1 nm/min. CD spectra are reported as $\Delta\epsilon$ per amide. The protein concentration was in the range of 0.5–1 mg/ml as determined on a Beckman amino acid analyzer. The secondary structure content was determined according to the method of Manavalan and Johnson (32).

Structural models were constructed using the Quanta-Charmm program by MSI Ltd. and Insight II-Discover software from MSI Technologies, Inc. (United Kingdom) running on a Silicon Graphics VGX R4000 Crimson Workstation. Models were built by the Homology module of Quanta on the basis of 59.4% identity to AaHII neurotoxin (10). The structures were minimized *in vacuo* using the Charmm force field. One hundred steps of minimization were performed using the steepest descent algorithm, followed by 5000 steps using the conjugate gradient method until a root mean square deviation of 0.001 was obtained. Electrostatic potentials of the unmodified and mutant toxins were calculated using Delphi software (MSI Technologies, Inc. (UK)).

RESULTS

Selection of sites to be modified was based on comparison between two homologous α -toxins, AaHII and Lqh α IT, displaying two extremes in their phylogenetic preferences. AaHII, the strongest anti-mammalian scorpion α -neurotoxin (6, 33), and Lqh α IT, the most insecticidal toxin among the α -group (5, 6, 22), compete very poorly with each other for their binding sites

on insect or mammalian sodium channels. This pharmacological difference can be attributed to nonhomologous residues or structural motifs located on their surfaces. Site-directed modifications were introduced to Lqh α IT to elucidate the molecular surface involved in recognition of the receptor site on the sodium channel. All toxin variants were purified by high performance liquid chromatography using the toxicity assay on blowfly larvae as a quick and direct measure of activity. Binding assays to a cockroach neuronal membrane preparation were used as a measure of direct activity at the receptor site. Possible structural alterations were assessed by CD spectroscopy.

Modification of Tight Turns—Comparison between the three-dimensional structures and amino acid sequences of AaHII (10) and Lqh α IT (5, 25) revealed major differences in the five-residue turn (residues 8–12) and in the C-terminal region: (i) residues Asp⁸-Asp⁹-Val¹⁰ in AaHII are located on an external region of the toxin surface and are conserved in a large group of α -toxins, whereas these positions are occupied by Lys⁸-Asn⁹-Tyr¹⁰ in Lqh α IT (22, 28) (Fig. 1); (ii) the disposition of the C termini relative to the five-residue turns shows a clear difference between Lqh α IT and AaHII (10, 25).

We initiated the mutagenesis program by substituting residues Lys-Asn-Tyr (positions 8–10) into Asp-Asp-Val (as appear at these positions in AaHII) and produced the recombinant Lqh α IT variant using our expression-reconstitution system (5). This modification affected the activity of the toxin dramatically: the apparent affinity for the insect receptor site decreased 21,000-fold, and the toxicity was practically lost (Fig. 2A, Table I). To determine whether this detrimental effect was due to a conformational change of the overall structure, the mutant toxin was analyzed by CD spectroscopy (Fig. 3A). Despite some changes in the spectrum, the calculated secondary structure content (32) was similar to that of the unmodified toxin. This result suggested that the mutation might have generated a local effect on the active molecular surface.

To identify the specific residue responsible for the detrimental effect, the following separate substitutions were conducted: (i) K8A and K8D to determine the significance of the positive charge at this position; and (ii) Y10S and Y10W to clarify whether a hydroxyl or an aromatic ring is crucial at this site. Neutralization of the positive charge at position 8 (K8A mutant) resulted in a 39.4-fold decrease in the apparent affinity for the receptor site on cockroach sodium channels and a 4-fold decreased toxicity to blowfly larvae (Fig. 2A, Table I) without a significant change in the CD spectrum (Fig. 3B). However, a severe effect was obtained when the charge at position 8 was inverted by replacing lysine with aspartate (K8D). The apparent affinity for the receptor site decreased 1611-fold, and less than 1% of the residual toxicity was determined (Fig. 2A, Table I). CD spectrum analysis of the K8D variant revealed a complete loss of the 190-nm band (Fig. 3A), suggesting changes in

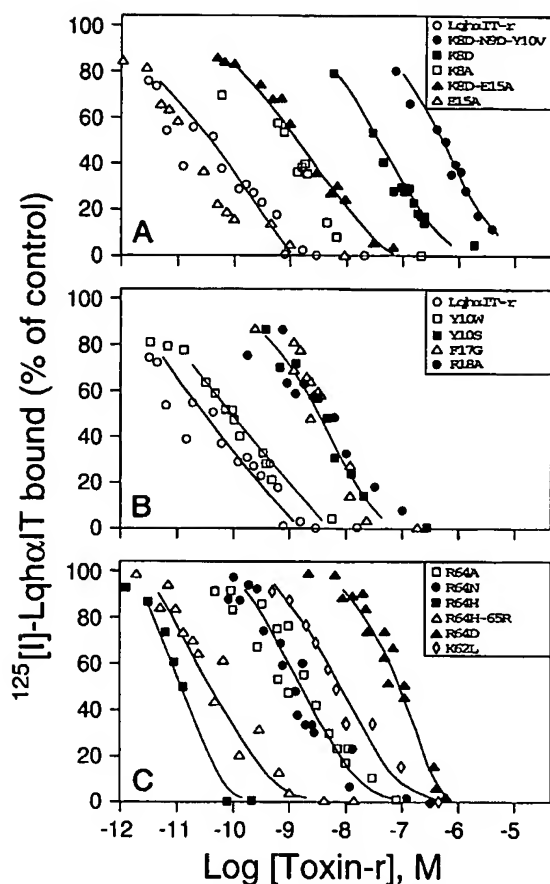


FIG. 2. Competitive inhibition of the binding of unmodified 125 I-labeled Lqh α IT by various recombinant Lqh α IT toxins. The unmodified toxin is compared with mutants modified in turn structures (A), mutants modified at residues presumably interacting with the receptor site (B), and mutants modified at the C-terminal region (C). Cockroach neuronal membranes (25 μ g of membrane protein) were incubated for 60 min at 22°C in the presence of 0.03 nM 125 I-labeled Lqh α IT and increasing concentrations of unmodified or mutant toxins. Nonspecific binding of 125 I-labeled Lqh α IT, determined in the presence of 1 μ M Lqh α IT (corresponding to 15–25% of total binding), was subtracted. The binding was determined as described (see “Experimental Procedures” and Ref. 6) and analyzed by the LIGAND computer program.

its secondary structure (see “Discussion”).

In an attempt to visualize the impact of the charge inversion, we compared the calculated electrostatic potentials of the unmodified, the K8D, and K8D/N9D/Y10V mutant toxins (Fig. 4). The unmodified toxin was found to be highly polar. One pole, containing the N terminus, was negatively charged, and the other pole, containing Lys⁸, Arg¹⁸, Arg⁵⁸, Lys⁶², and the C-terminal Arg⁶⁴, was positively charged (Fig. 4, top left). The “bullet-shaped” electrostatic potential was severely disrupted in mutant K8D (Fig. 4, top right) and only mildly disrupted in mutant K8A (data not shown). We investigated whether this disruption was due to the charge inversion at position 8, causing a conformational change (Fig. 3A), or to alteration of the overall charge distribution of the molecule by neutralizing the negative charge of the adjacent Glu¹⁵ (E15A mutant). This substitution was based on results obtained by Delphi calculations with K8D and K8D/E15A computer models suggesting a detrimental effect on the electrostatic potential caused by two adjacent negative charges, *i.e.* Asp⁸ and Glu¹⁵ (Fig. 4, top right). The E15A mutation alone revealed no significant change in the binding affinity, a 4-fold decrease in toxicity (Table I, Fig. 2A), no change in the CD spectrum (Fig. 3A), and a nonsignif-

TABLE I
Biological activity of the recombinant toxins

Toxin	ED ₅₀ ^a	Toxicity	IC ₅₀ ^b	Increase in IC ₅₀
	ng/100 mg body weight	%	nM	-fold
Unmodified	12.8	100	0.035 \pm 0.01	1
K8D/N9D/Y10V	3500	0.4	745.6 \pm 130.5	21,303
K8D	2100	0.6	56.4 \pm 11.0	1,611
K8A	50	26	1.38 \pm 0.41	39.4
Y10W	30	43	0.13 \pm 0.04	3.7
Y10S	129	10	4.0 \pm 0.6	114.3
K8D/E15A	238	5	2.5 \pm 0.5	71.4
E15A	55	23	0.021 \pm 0.013	0.6
F17G	92	14	4.6 \pm 0.9	160
R18A	59	22	8.1 \pm 1.9	231.4
D19N	11	115	0.043 \pm 0.01	1.2
D19H	52	25	0.21 \pm 0.1	6
R18K/D19N	31	41	0.016 \pm 0.008	0.46
G40S/K41P	20	64	0.9 \pm 0.3	25.7
K62L	69	19	7.7 \pm 3.1	220
R64N	12	106	2.05 \pm 0.7	58.6
R64D	258	5	83.8 \pm 26.0	2,394
R64A	24	53	1.9 \pm 0.8	54.3
R64H	4	320	0.012 \pm 0.009	0.33
R64H/65R	28	46	0.06 \pm 0.03	1.7

^a ED₅₀ was determined on blowfly larvae (100 \pm 20 mg).

^b IC₅₀ was determined on cockroach neuronal membrane preparations.

icant change in the electrostatic potential (data not shown). However, an E15A mutation in addition to the K8D mutation (K8D/E15A double mutant) had no effect on the CD spectrum, remaining similar to that of mutant K8D (Fig. 3A), but restored the electrostatic charge distribution to a great extent (Fig. 4, top left). This restoration was reflected by substantial increases in the apparent affinity for the receptor site (22-fold) and toxicity (8.3-fold) of the double mutant compared with the K8D mutant (Fig. 2A, Table I). These results suggest that the electrostatic potential may be important for the biological activity of Lqh α IT.

The possible role of the aromatic Tyr¹⁰, belonging to the five-residue turn, was investigated by two substitutions. A Y10W substitution had a minute effect on toxicity (Fig. 2B, Table I). However, replacement by serine caused a 114-fold decrease in the apparent affinity for the receptor site (Fig. 2B, Table I), and only 10% of the toxicity was detected (Table I). In light of the unchanged CD spectra of mutants Y10S (Fig. 3B) and Y10W (not shown), these results suggest that the aromatic side chain at position 10 may interact with the receptor site.

The 40–43 β -turn is unique to α -scorpion neurotoxins and thus could pertain to their characteristic pharmacology (19). In other α -toxins, residues 40 and 41 are serine and proline, respectively, whereas glycine and lysine occupy these positions in Lqh α IT. Still, the G40S/K41P mutation had a mild effect on toxicity. Although the 190-nm band decreased substantially in the CD spectrum of this mutant (Fig. 3C), the apparent affinity for the receptor site decreased only 25.7-fold, and 64% of the toxicity was preserved (Table I). These results suggest that this tight turn does not play a critical role in the insecticidal activity of Lqh α IT.

Modification of the Loop Preceding the α -Helix—From the solution structure of Lqh α IT (25) it was apparent that residues Phe¹⁷ and Arg¹⁸ in the loop preceding the α -helix (residues 19–28) belong most likely to the molecular surface common to the aforementioned turns. Phe¹⁷ is unique to Lqh α IT, and Arg¹⁸ is found at this position in most α -neurotoxins with high activity against mammals (Fig. 1) (6, 34). Phe¹⁷ was replaced by glycine, found in most α -toxins, and Arg¹⁸ was substituted by alanine to examine the role of its charge. The IC₅₀ of mutant F17G was affected markedly (160-fold increase), and 14% of the

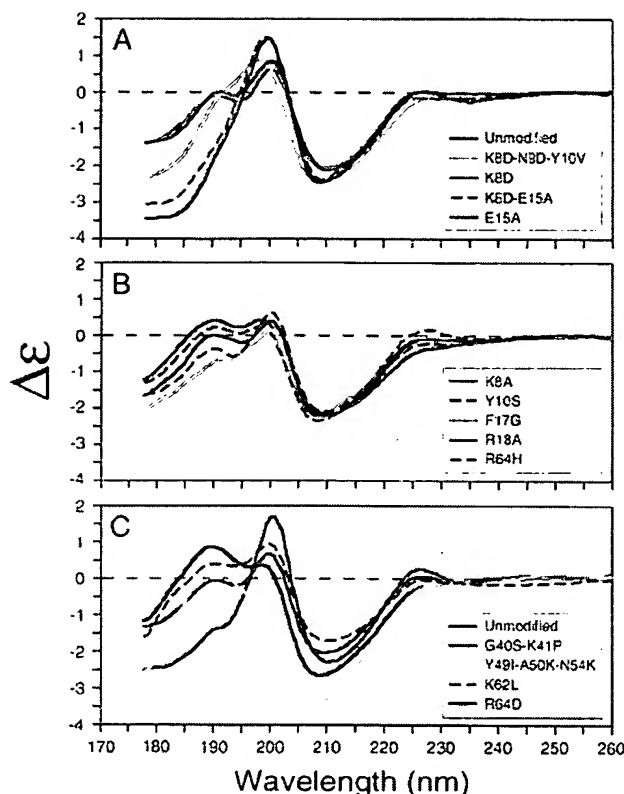


FIG. 3. Circular dichroism spectra of unmodified and mutant Lqh α IT toxins. The unmodified toxin is compared with mutants modified at the five-residue turn and a suppressor mutant (A), mutants with minor structural changes (B), and mutants modified at the 40–43 β -turn and at the C-terminal region (C). Spectra of some mutants that resembled that of the unmodified toxin, i.e. Y10W, D19N, D19H, R18A/D19N, R64N, and R64HR, were omitted.

toxicity was determined (Fig. 2B, Table I). The apparent affinity of mutant R18A for the receptor site on the cockroach sodium channels decreased 231-fold, and 22% of the toxicity was determined (Fig. 2B, Table I). However, only minor changes in calculated electrostatic potentials (data not shown) and CD spectra (Fig. 3B) were observed.

The residue adjacent to Arg¹⁸ is the first amino acid in the α -helix. In Lqh α IT, this position is occupied by aspartate (Asp¹⁹), whereas asparagine is found at this position in most α -neurotoxins (Fig. 1). Mutant D19N revealed no change in the apparent affinity for the receptor site but some increase in toxicity to blowfly larvae (Table I). Mutant D19H revealed a slight decrease in activity. A double mutant, R18K/D19N, exerted some improved binding affinity but a slight decrease in toxicity (Table I). Apparently, the negative charge at position 19 has no important role in the toxicity of Lqh α IT.

Modifications of the C Terminus—The region connecting Cys¹² (five-residue turn) with Cys⁶³ (C-terminal region) by a disulfide bridge was shown to participate in an immunoreactive epitope whose blocking by specific antibodies inhibited the binding of AaHII to rat brain synaptosomes. Furthermore, Val¹⁰, Lys⁵⁸, His⁵⁴, and/or His⁶⁴ were proposed to play a role in the interaction with a monoclonal antibody, which precluded binding of AaHII to the receptor site (35).

The possible involvement of the C terminus in the toxic site of Lqh α IT was investigated by modifying the terminal Arg⁶⁴ and the adjacent Lys⁶². The positive charge of Arg⁶⁴ was either neutralized (R64A, R64N) or inverted (R64D). Furthermore, the terminal Arg⁶⁴ was substituted by histidine, found at the C terminus of several other α -toxins, and by a histidine-arginine

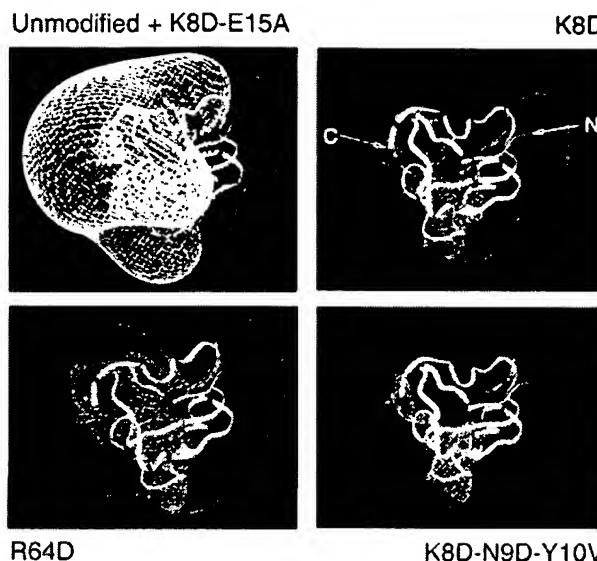


FIG. 4. The electrostatic potentials of Lqh α IT and modified toxins. The positive (+1 kcal/mol) surface of the unmodified toxin is shown as a white net (top left), and the positive surfaces of the mutants are shown by blue nets. The negative (−1 kcal/mol) surfaces are indicated by the red nets or by the purple net in the double mutant K8D/E15A. The structures and electrostatic potentials of the unmodified and K8D/E15A double mutant are superimposed. N and C stand for the N and C termini, respectively. The orange ribbons, oriented similarly in all variants, indicate the carbon backbones.

pair, as found in the cDNA sequence of Lqh α IT (28). Lys⁶² was converted to leucine to assess the significance of its positive charge. As shown in Fig. 2C and Table I, the apparent affinity for the receptor site of mutants R64A and R64N decreased, whereas their toxicity (Fig. 2C, Table I) and CD spectra remained practically unchanged (not shown). Orientation of their electrostatic potentials did not change, albeit there was a reduction in the overall positive charge (data not shown). Mutant R64D, however, revealed a marked decrease in the apparent affinity for the receptor site and 20-fold reduced toxicity (Table I, Fig. 2C). These effects were in concert with a marked disruption of the electrostatic potential (Fig. 4) and a change in CD spectrum (Fig. 3C). Another severe effect occurred with mutant K62L (220-fold decrease in apparent binding affinity and 5-fold reduced toxicity) (Table I, Fig. 2C). This effect was accompanied by an increase in the 190-nm band of the CD spectrum (Fig. 3C) and a marked decrease in the positive component of the electrostatic potential (not shown).

In contrast to the mutants showing reduced activity, mutant R64H revealed a 3.2-fold increase in toxicity and a 3-fold improved binding affinity for the receptor site (Fig. 2C, Table I). The CD spectrum of this variant remained similar to that of the unmodified toxin (Fig. 3B).

All toxin variants were injected to mice in parallel with the biological tests on insects. Interestingly, the changes in the insecticidal activity of each of the mutant toxins correlated with the changes in the anti-mammalian toxicity (not shown).

DISCUSSION

The genetic approach in the present study has become possible due to an efficient expression-reconstitution system established recently for the highly insecticidal scorpion α -neurotoxin Lqh α IT and its genetic variants (5). Interestingly, the yields of functional polypeptide variants were similar to that obtained for the unmodified toxin, varying between 2 and 5 mg of protein per liter of *E. coli* culture, and no correlation between

the yield, bioactivity, and/or CD spectrum was observed. Point mutagenesis highlighted amino acid residues having a role in receptor site recognition, structural arrangement, and formation of a polar electrostatic surface.

A Putative Dual Role for Lys⁸—Since variations in animal group specificity among toxins belonging to the same neuropharmacological class are most likely related to structural differences at their putative toxic sites, we directed the initial modifications to such a variable motif, namely the five-residue turn of Lqh α IT. This turn (residues 8–12) in Lqh α IT (KYNVC), the most insecticidal scorpion α -toxin (6, 22), differs greatly from its corresponding turn in AaHII (DDVNC), the strongest anti-mammalian scorpion α -neurotoxin (6, 33). The profound decrease in toxicity and binding affinity to the receptor site caused by the K8D/N9D/Y10V mutation (Table I) suggested a major role for residues 8–10. At this point it was important to clarify whether the K8D/N9D/Y10V mutation affected the overall structure, which could consequently lead to loss of activity, or whether it was a local effect on residues exposed to the solvent and, thereby, disrupted direct interactions with the receptor binding site. CD spectrum (Fig. 3A) and singular value decomposition-derived data analyses of the mutant toxin revealed a secondary structure content similar to that of the unmodified toxin, suggesting preservation of the overall spatial arrangement of the mutant toxin. The modifications performed with either Lys⁸ or Tyr¹⁰ were made to determine the specific residue responsible for the detrimental effect obtained with the triple mutation. The marked decrease in the apparent binding affinity and toxicity obtained with the K8D mutation (Table I) implied a major role for this residue. This detrimental effect could be due to either direct disruption of a structural motif or indirect alteration generated by repulsion between Asp⁸ and Glu¹⁵, elimination of a direct interaction with a recognition point at the receptor site, and/or alteration of the polarity of the molecule. Analysis of mutants K8A, E15A, and K8D/E15A provided data enabling discrimination among these possibilities. The importance of a positive charge at this position was shown by the decrease in toxicity of the K8A mutant (Table I). Since the electrostatic potential and CD spectrum of the K8A mutant changed only slightly, the decrease in activity did not result from disruption in secondary structure or from elimination of the putative interaction with the adjacent Glu¹⁵. It is more likely that Lys⁸ interacts electrostatically with the receptor site, and since this interaction is part of a multipoint interacting surface (31, 36), its effect is rather slim.

However, inversion of the charge at position 8 (K8D mutant) caused a severe effect on biological activity (Table I) in addition to a complete disappearance of the 190-nm band in the CD spectrum (Fig. 3A). This band was found to correlate with either α -helix and/or turn structures (37). We speculate that the changes observed in the 190-nm band in the CD spectra of mutants K8D and G40S/K41P are associated with structural alterations of tight turns alone. That is because the modifications were introduced to turn structures distant from the single α -helix of this molecule. On the basis of the structural difference between AaHII (10) and Lqh α IT (25), we speculate that this structural change might cause Asp⁸ to form hydrogen bonds, as is found in AaHII (10), leading consequently to a buried position in the mutant K8D. Furthermore, the electrostatic potential of this variant was disrupted as well (Fig. 4). Restoration of activity obtained by the E15A suppressor mutation while the CD spectrum remained similar to that of the K8D mutant (Fig. 3A) suggested that neither the change in secondary structure nor the negative charge at position 8 were fully responsible for the detrimental effect caused by mutation K8D. The unchanged CD spectrum of mutant K8D/E15A com-

pared with that of mutant K8D also contradicted the possibility that a repulsion between Asp⁸ and Glu¹⁵ caused the alteration in secondary structure (Fig. 3A). It is possible that introduction of two negative charges (Asp⁸ and Glu¹⁵) at the positive pole disrupts the electrostatic potential of the molecule, whereas one negative charge (Glu¹⁵ in the unmodified toxin, provided that no interaction with Lys⁸ exists, or Glu¹⁵ in the K8A mutant, or Asp⁸ in the K8D/E15A mutant) is not detrimental. This conclusion is supported by the results obtained with the K8D/N9D/Y10V triple mutant. Three negative charges (Asp⁸, Asp⁹, and Glu¹⁵) changed the electrostatic potential of the toxin (Fig. 4); indeed, the apparent affinity for the receptor site decreased 21,000-fold, and only 0.4% of the residual toxicity was measured (Table I). These results may suggest an important role for the electrostatic potential in the biological activity of the toxin.

Comparison between the structures of the five-residue turns in AaHII (10) and Lqh α IT (25) provides further insights regarding the critical effect obtained by the K8D substitution. In AaHII, hydrogen bonds exist between Asp⁸ and each of the residues Cys¹², Val¹⁰, and Asn¹¹ (10). This network stabilizes the five-residue turn and may influence the C-terminal stretch through the Cys¹²–Cys⁶³ disulfide bridge. In Lqh α IT, however, Lys⁸ is exposed to the solvent, in contrast to the buried Asp⁸ in AaHII. Thus, stabilization of the five-residue turn in Lqh α IT is achieved differently. From the solution structure of Lqh α IT (25), it is apparent that Tyr¹⁰ interacts most likely with the terminal Arg⁶⁴, and Lys⁸ does not seem to interact with Glu¹⁵ but rather with Tyr¹⁴. As mentioned above, the lack of interaction between Lys⁸ and Glu¹⁵ reflects the negligible change in the CD spectrum obtained with mutant E15A (Fig. 3A). Thus, Lys⁸, whose substitution by alanine had a moderate effect on toxicity, may play a dual role by interacting electrostatically with the receptor site and contributing to the polar electrostatic surface of Lqh α IT.

Aromatic Residues in the Putative Toxic Surface—The importance of the aromatic side chain of Tyr¹⁰ for toxicity was apparent from the unchanged toxicity of mutant Y10W compared with mutant Y10S, which revealed a 110-fold decrease in the apparent binding affinity for the receptor site (Table I, Fig. 2B). The possible interactions of Tyr¹⁰ with the C-terminal arginine, inferred from the solution structure of Lqh α IT (25), may suggest that the precise position of Tyr¹⁰ is important and needs special stabilizing interactions.

Another aromatic residue showing importance for toxicity is Phe¹⁷. This is inferred from the substantial decrease in biological activity (Table I) without a significant change in CD spectrum (Fig. 3B) upon its substitution. Its location within the putative molecular surface common to the five-residue turn and the C terminus suggests that Phe¹⁷ may interact directly with the receptor site.

Examination of the spatial arrangement of Lqh α IT suggests that the side chains of additional aromatic amino acids, such as Tyr¹⁴, Tyr²¹, or Trp³⁸, are located in the vicinity of the putative toxic surface and may be important for toxicity. In accordance with this notion is the result obtained by sulfenylation of Trp³⁸ in AaHII, which decreased the toxicity and apparent binding affinity for mammalian sodium channels by more than 1 order of magnitude, whereas the CD spectrum of the modified toxin remained unchanged (36).

Conversely, modifications of two aromatic residues that belong to the conserved hydrophobic surface, *i.e.* sulfenylation of Trp⁴⁵ in AaHII (the equivalent of Trp⁴⁷ in Lqh α IT) and substitution of Tyr⁴⁹ by isoleucine in Lqh α IT (5), had a negligible effect on toxicity to mammals and insects, respectively. In both instances, the CD spectra of the modified toxins changed dramatically (Ref. 36 and data not shown). Thus, it is likely that

the conserved hydrophobic surface may not be involved in the toxic surface.

Charged Residues in the Putative Toxic Surface—Our results pinpoint several charged residues, in addition to Lys⁵⁸ (see above), that show significance for activity. One such residue is Arg¹⁸, as shown by the R18A mutation. The CD spectrum of this mutant (Fig. 3B) was similar to that of the unmodified toxin, and its electrostatic potential was only moderately affected (data not shown). Therefore, the decrease in activity of mutant R18A (Table I) may suggest a direct electrostatic interaction of Arg¹⁸ with the receptor site. Interestingly, substitution of the adjacent Asp¹⁹ by asparagine had no effect on toxicity, and a D19H mutation affected the activity only moderately. The double mutant R18K/D19N also had a minor effect (Table I). These results imply that residue 19, the first residue of the α -helix stretch (residues 19–28), may have no role in the interaction with the receptor site. This result is in concert with the finding that antibodies raised against the α -helix region of AaHII and the turn comprising residues 27–30 were capable of binding to the toxin when associated with the receptor site (14).

Superposition of the structures of AaHII and Lqh α IT reveals also that the C termini of both toxins are positioned between the five-residue turn and the β -turn formed by residues 40–43. However, their dispositions relative to the five-residue turns differ in both toxins. Based on these observations, we modified Lys⁶² and Arg⁶⁴ to investigate their contribution to the putative toxic surface. Neutralization of the positive charge of the C-terminal residue (R64A or R64N) decreased the apparent affinity for the receptor site more than 50-fold, although the toxicity remained practically unchanged (Table I). The discrepancy between the effects obtained *in vivo*, using blowfly larvae, and *in vitro*, using cockroach neuronal membranes, may be related to the inherent differences between fly and cockroach sodium channel structures. Differences in sodium channel proteins of various insect central nervous system membranes have been shown at the biochemical level (38, 39). Moreover, the ability of scorpion neurotoxins to distinguish among sodium channel preparations of various insects has also been reported (6, 24, 39). The similarity between the CD spectra of mutant R64N (not shown) and that of the unmodified toxin minimized the possibility that mutation R64N caused a structural change affecting activity. More likely is the suggestion that Arg⁶⁴ interacts electrostatically with the receptor site.

Inversion of the charge at the C terminus, mutation R64D, had a dramatic effect: the apparent binding affinity decreased 2394-fold, and the remaining toxicity was 5% (Table I). Such a detrimental effect can result from a conformational alteration, as inferred from the altered CD spectrum (Fig. 3C), the change of the electrostatic potential of the toxin (Fig. 4), or the perturbation of the electrostatic contact with the receptor site. Except for the disruption of the contacts between Arg⁶⁴ and Tyr¹⁰, Asp⁶⁴ may generate a new intramolecular contact with a nearby residue, leading to some structural change, as was suggested by the altered CD spectrum of this mutant (Fig. 3C). Yet, this putative alteration, reflected by the highly sensitive measurement of the CD spectrum, had only a minute effect on the energy-minimized α -carbon model of the mutant toxin (Fig. 4).

Another positive charge related to the C-terminal domain is Lys⁶². Its putative interaction with the receptor site is shown by the 220-fold decrease in the apparent binding affinity to the cockroach sodium channel when substituted by leucine (Table I). The moderate change observed in the CD spectrum of mutant K62L (Fig. 3C) may suggest that this residue is involved in an intramolecular interaction as well.

In a previous study, biotinylation of Lys⁵⁸ of AaHII resulted

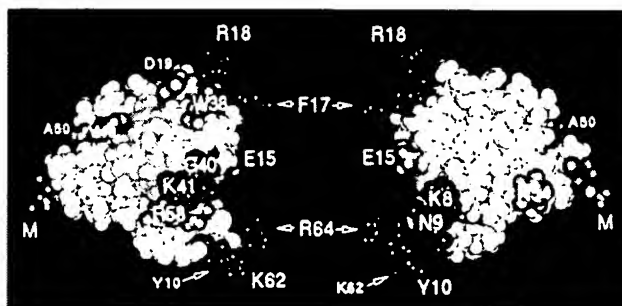


FIG. 5. The putative toxic surface of Lqh α IT. The model was constructed according to the solution structure of the toxin (25). Genetically modified residues affecting the toxic surface are designated in red. Residues whose counterparts in similar toxins were affected by chemical modifications are shown in orange. Residues whose substitution had no effect are indicated in green. The conserved hydrophobic surface, located on a different side of the molecule, appears in blue. Orientation of the carbon backbone is to a similar direction as in Fig. 4 with some variations for better visualization.

in the most devastating effect obtained with chemical agents applied on scorpion toxins affecting sodium channels (16). Only 1% of the original toxicity toward mammals was determined, and no detectable displaceability of the native toxin from the mammalian receptor site could be monitored with the modified toxin. From the known structure of AaHII (10), Lys⁵⁸ was suggested to interact with Asn¹¹ and Gly⁶¹ by hydrogen bonding. This network may be important for stabilizing the C terminus relative to the five-residue turn. By analogy to Lqh α IT and its putative toxic surface, we speculate that the prominent effect obtained in the biotinylated AaHII could be due to a combination of structural disruption, elimination of a putative electrostatic interaction with the receptor site, and/or alteration of the electrostatic potential of the molecule.

Conclusion—Our study pinpoints a putative molecular surface involved in recognition of the receptor site that differs from the conserved hydrophobic surface located on a different side of the molecule (Fig. 5). The plausible suggestion of a multipoint interaction of scorpion α -neurotoxins with their binding sites (31, 36) is in concert with our results, in which each substitution of a residue participating in binding has a partial effect on activity, whereas a structurally devastating mutation may cause a more severe effect.

Binding to the receptor site may involve electrostatic as well as hydrophobic interactions with opposing constituents at the receptor site. This was demonstrated in other toxin receptor site interactions, e.g. K⁺ channels and short scorpion neurotoxins such as charybdotoxin, leiurotoxin, and PO5 (40–43), or the nicotinic acetylcholine receptor and a sea snake toxin, erabutoxin α (44). The most recent finding, showing that inversion of a negative glutamate into a positive residue in the extracellular linker between sodium channel transmembrane segments S3 and S4 in domain IV disrupts the binding of an α scorpion neurotoxin to rat brain sodium channels (45), is in concert with our suggestion. The significance of the electrostatic potential for the binding of a short scorpion toxin, Lq2, to its receptor site was inferred from the inversion of a negatively charged residue in a potassium channel, which affected association rather than the dissociation rate of the toxin (46). From the correlation between the changes in biological activities and alterations of the putative electrostatic potentials of the various modified toxins produced in the present study, the electrostatic potential seems to play a role in the biological activity of the long scorpion neurotoxin Lqh α IT. Whether this polarity is related to association or dissociation kinetics of the toxin is still unknown and deserves further study.

An alternative approach to the elucidation of the toxic site of neurotoxins affecting the cholinergic binding site was demonstrated by a combination of site-directed mutagenesis of isolated fragments of the receptor (47, 48) and NMR analyses of bound ligands (49).

Three types of animals, *i.e.* fly larvae, cockroaches, and mice, were used in the bioassays. Injection to fly larvae provided a useful and quick measure of the effects caused by the modifications and throughout the purification process of each of the toxin variants. However, the effects of the mutations on the apparent binding affinity to the cockroach central nervous system sodium channels were in most instances more intense than the effects on toxicity to fly larvae. This phenomenon may result from (i) differences in sensitivity of various insect sodium channels from different tissues or animals to Lqh α IT and to its modified toxin (6) or (ii) variations in susceptibility of the toxin variants to proteolysis or availability to various tissues in the whole animal that result from the chemical features of the toxin, *i.e.* hydrophobicity or change in charge. Hence, binding-competition studies using purified neuronal membranes are a direct measure for toxin receptor site interactions on sodium channels and thus provide a more sensitive assay than toxicity assays on whole animals.

The similar changes in toxicity toward fly larvae and mice obtained with the various mutants suggest that their corresponding receptor sites are similar. Further modifications of residues that belong to the putative recognition site may be useful for final determination of the toxic site and for clarifying the molecular basis of animal group specificity of scorpion α -toxins.

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Molecular Mimicry Between the Rabies Virus Glycoprotein and Human Immunodeficiency Virus-1 GP120: Cross-Reacting Antibodies Induced by Rabies Vaccination

By Luisa Bracci, Samir K. Ballas, Adriano Spreafico, and Paolo Neri

The 160-170 sequence of human immunodeficiency virus (HIV)-1 gp120 mimics a nicotinic receptor-binding motif of rabies virus glycoprotein and snake neurotoxins. This sequence has been proposed to be involved in the binding of HIV-1 gp120 to the acetylcholine binding sites of nicotinic receptors. By using biomolecular interaction analysis (BIA) technology we have found that HIV-1 gp120 can bind to detergent-extracted nicotinic receptor from fetal calf muscle. The binding is inhibited by nicotine and by a synthetic peptide reproducing the gp120 160-170 sequence. The molecular mimicry between gp120 and rabies virus glycoprotein is confirmed by cross-reacting antibodies. We have found that vac-

cination against rabies can induce the production of anti-HIV-1 gp120 antibodies in humans. The cross-reacting antibodies are directed to the gp120 sequence involved in the mimicry with the rabies virus glycoprotein. The cross-reactivity between the rabies virus and HIV-1 has important implications in transfusion medicine. Moreover, the presence of cross-reacting antibodies between the nicotinic receptor binding site of rabies virus glycoprotein and a fragment of HIV-1 gp120 strengthens the hypothesis about the possible role of nicotinic receptors as potential receptors for HIV-1 in the central nervous system.

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THE SEQUENCE 160-170 of human immunodeficiency virus (HIV)-1 envelope protein gp120 is highly homologous to a sequence shared by snake curare-mimetic neurotoxins and the rabies virus glycoprotein.¹ This sequence is involved in the specific binding of rabies virus glycoprotein and snake neurotoxins to the nicotinic acetylcholine receptor (AChR) at neuromuscular junctions.²⁻⁵

In previous reports,^{1,6} we proposed that the mimicry of a nicotinic receptor binding motif by HIV-1 gp120 can be important in HIV-1 infectivity by enabling HIV-1 binding to the nicotinic receptor acetylcholine binding sites. This hypothesis was supported by experimental data indicating that recombinant gp120 from HIV-1 IIIB can inhibit the binding of the snake neurotoxin α -bungarotoxin (α -bgt) to the nicotinic receptor in a human rhabdomyosarcoma cell line.⁶ Moreover, we showed that immunization of mice with a synthetic peptide reproducing the sequence 160-170 of HIV-1 gp120 induces the production of antibodies cross-reacting with rabies virus glycoprotein and α -bgt.⁶ Taken together, these data indicate that a region of HIV-1 gp120 is structurally and functionally related to an important functional site of rabies virus glycoprotein.

Apart from the possible relevance in HIV-1 infectivity, it is reasonable to expect that this similarity can cause the production of cross-reacting antibodies. Our hypothesis is supported by a report⁷ describing a false-positive HIV-1 screening related to rabies vaccination. The investigators reported a case of a woman who had been vaccinated against rabies and soon after the last boost gave a positive result in an enzyme immunoassay (EIA) for anti-HIV-1 antibodies. The woman was a blood donor, and she had been monitored for the presence of anti-HIV-1 antibodies and she tested negative in the EIA until a few months before the antirabies vaccination. Fifteen days after the last injection of the rabies vaccine, a repeatedly positive EIA occurred. At the same time a polymerase chain reaction for proviral DNA was negative. The EIA for anti-HIV-1 antibodies was negative again 6 weeks after the first positive result.

We have tested different serum samples of the described patient in enzyme-linked immunosorbent assay (ELISA), and we have found a clear cross-reactivity with HIV-1 recombinant gp120 and with a synthetic peptide reproducing the rabies virus glycoprotein-homologous sequence of HIV-

1 gp120. Moreover, we have analyzed the binding of HIV-1 gp120 to detergent-extracted nicotinic receptor from fetal calf muscle membranes by surface plasmon resonance (SPR). We have found that a component from fetal calf muscle membranes binds to HIV-1 gp120. The binding is inhibited by nicotine and by the synthetic peptide, which reproduces the snake neurotoxin and rabies virus glycoprotein mimicking sequence of HIV-1 gp120.

MATERIALS AND METHODS

Peptide synthesis. Peptide B2 (C-S-F-N-I-S-T-S-I-R-G-K-V-Q-K-E) was synthesized by solid phase synthesis using a MultiSynTech model "Syro" multiple peptide synthesizer (Bochum, Germany) employing fluorenylmethoxycarbonyl (Fmoc) chemistry. Fmoc amino acid derivatives were coupled by using N-hydroxybenzotriazole-esters and each coupling step was monitored by the bromophenol blue acid-base indicator. Peptides were cleaved from the resin and simultaneously deprotected using a 93% trifluoroacetic acid/2% anisole/3% ethanedithiol/2% water mixture. The crude products were gel-filtrated on Sephadex G10 and purified by reverse-phase high performance liquid chromatography (HPLC). Purified peptides were controlled by capillary electrophoresis (Biofocus 3000; Bio-Rad Laboratories, Hercules, CA) and by amino acid composition and sequence analysis.

ELISA on recombinant gp120. EIA plates (96-well) were coated with 2 μ g/mL of recombinant gp120 (IIIB, Intracel, London, UK) in 50 mmol/L carbonate buffer, pH 9.6, overnight at 4°C. Plates were washed after each step with phosphate-buffered saline (PBS),

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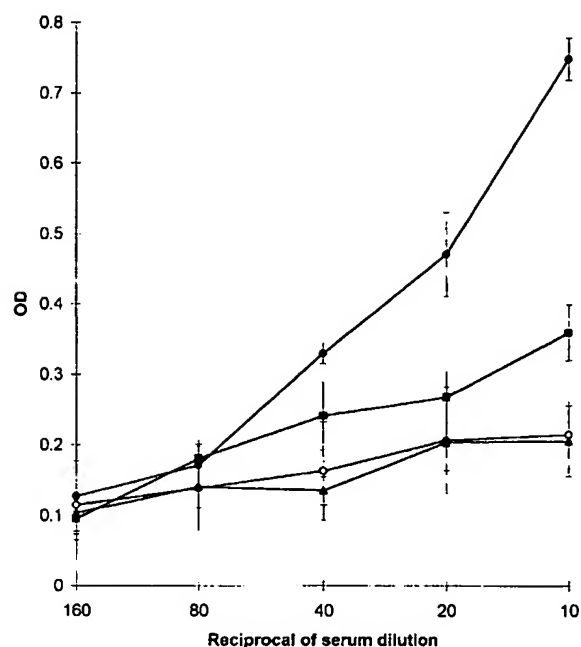


Fig 2. ELISA on recombinant HIV-1 gp120 of the serum from the rabies vaccinated patient. Serial dilutions of three samples of serum (RI [●], RII [■], and RIII [▲]), which had been collected at different times, were tested on EIA plates coated with recombinant gp120. Each point in the RI, RII, and RIII lines is the average of triplicate samples for each dilution of serum in two different experiments, while each point of the normal human sera (NHS [○]) line is the average of triplicate samples of 11 different HIV-negative sera at the same dilutions in two different experiments.

We used the BIAcore to study the interaction between HIV-1 gp120 and mammalian AChR solubilized from fetal calf muscle membranes. HIV-1 gp120 was covalently immobilized via primary amino groups on the dextran matrix, and detergent-solubilized nicotinic receptor prepared as described in Gotti et al⁸ was subsequently injected over the matrix. This experimental approach enabled us to detect the binding of gp120 to a component from the detergent-solubilized fetal calf muscle membranes. Competition experiments were done to test the specificity of the binding. The detergent-extracted fetal calf muscle membranes were injected over the matrix-coupled HIV-1 gp120 in the presence of nicotine. Using increasing concentrations of nicotine, we obtained up to 80% inhibition of the binding (Fig 6).

To test whether the binding of HIV-1 gp120 to the nicotinic receptor could be mediated by the gp120 sequence, which mimics the nicotinic receptor-binding motif shared by snake neurotoxins and rabies virus glycoprotein, the synthetic peptide B2 was tested for its ability to interfere with the binding of the nicotinic receptor to gp120 in BIAcore. This peptide was able to inhibit the binding of fetal calf nicotinic receptor to immobilized gp120. A synthetic peptide of the same length, but with a random sequence, had no effect on the binding (Fig 7).

DISCUSSION

The results reported seem to indicate that rabies vaccination can induce the production of antibodies cross-reacting

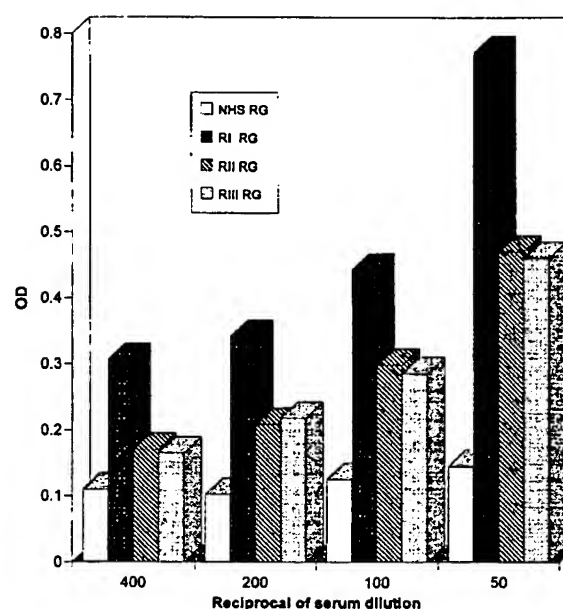


Fig 3. ELISA on rabies virus glycoprotein of the serum from the rabies vaccinated patient. Serial dilutions of three samples of the patient's serum (RI, RII, and RIII) collected at different times were tested on rabies virus glycoprotein using an EIA kit for the determination of antirabies virus glycoprotein antibodies.

with HIV-1 gp120. These cross-reacting antibodies decrease with time and can probably be detected only when the antirabies virus antibody titer is relatively high. Because a synthetic peptide reproducing the 157-172 sequence of HIV-1 gp120 is clearly recognized, both when used in solution and when immobilized on a ELISA plate, by serum antibodies from the rabies vaccinated patient, it seems that at least some of the cross-reacting antibodies are directed to this gp120 sequence, which is highly homologous to the nicotinic receptor-binding motif of rabies virus glycoprotein.

The reactivity of serum antibodies with the synthetic peptide B2 is decreasing with time. Nevertheless, an OD response higher than that we have with control HIV-1 negative sera is present even in the RIII sample of serum, which is negative in the ELISA on recombinant gp120. This can be explained by the high homology between the gp120 sequence reproduced in the B2 peptide and the corresponding rabies virus glycoprotein sequence. Due to this similarity, peptide B2 is probably recognized by a higher percentage of antirabies antibodies compared with the same sequence in the native gp120 conformation.

Table 1. Antirabies Virus Glycoprotein Titer of Serum Samples From the Rabies Vaccinated Patient

Serum Specimen	Date of Collection	Antirabies Virus Glycoprotein Titer
RI	8/92	2.0 UI/mL
RII	1/95	1.2 UI/mL
RIII	11/95	1.0 UI/mL

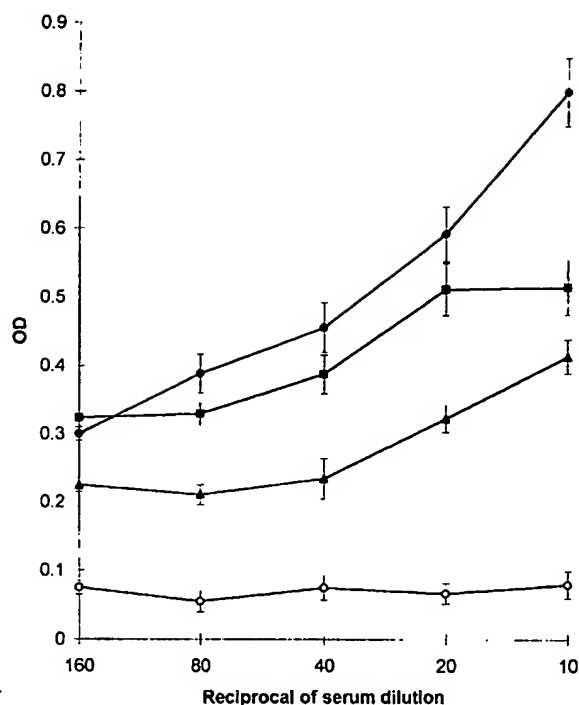


Fig 4. ELISA on the synthetic peptide B2 of the serum from the rabies vaccinated patient. Experimental conditions were the same as described in the Fig 2 legend. (RI (●), RII (■), RIII (▲), NHS (○)).

The antibody cross-reactivity between the rabies virus and HIV-1, which can be induced by rabies vaccination, has important implications in transfusion medicine. We are not aware of other cases of false-positive HIV tests related to

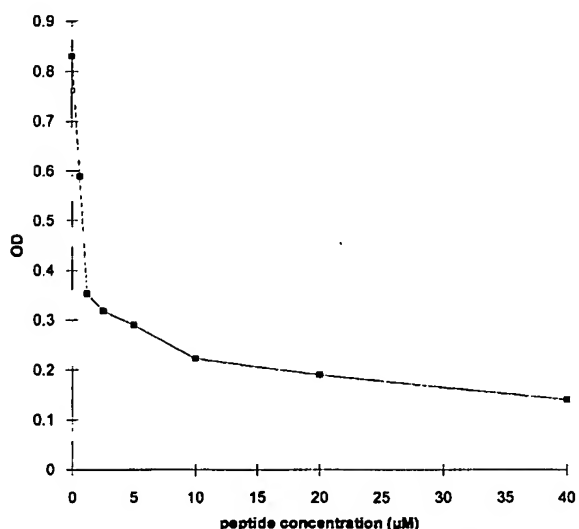


Fig 5. ELISA on gp120 of the serum from the rabies vaccinated patient in the presence of peptide B2. A 1:10 dilution of RI sample from the rabies vaccinated serum was mixed with increasing concentrations of peptide B2 and added to a gp120-coated EIA plate.

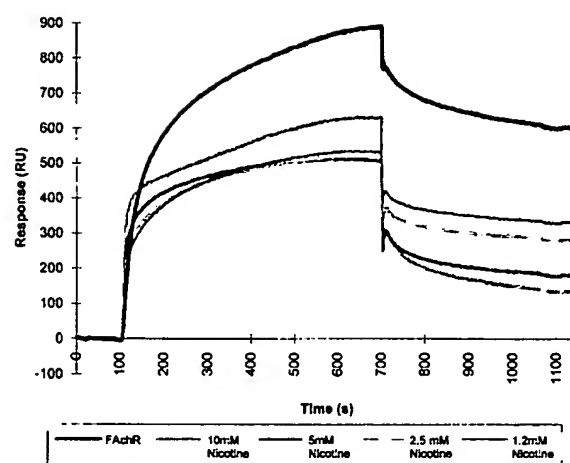


Fig 6. SPR of fetal calf muscle nicotinic receptor binding to HIV-1 gp120. Triton-extracted membranes from fetal calf muscle (FACHR) were diluted in 10 mmol/L acetate buffer, pH 6.5, 150 mmol/L NaCl to a concentration of 0.5 nmol of 125 I- α -bgt bound/liter and then injected at a flow rate of 5 μ L/minute over a matrix where HIV-1 gp120 had been covalently immobilized. The same sample was injected in the presence of increasing concentrations of nicotine. Regeneration of the matrix was obtained by injection of 10 mmol/L NaOH at the end of each cycle.

rabies vaccination. This is rather understandable considering the relatively low incidence of rabies vaccination in humans. Nevertheless, it would be interesting to test different sera from individuals who have been vaccinated against rabies, to see whether the cross-reaction of antirabies antibodies with HIV-1 gp120 is a common event.

The cross-reactivity of antirabies virus glycoprotein antibodies with HIV-1 gp120 confirms the data previously obtained through immunization of mice with the gp120 peptide B2: antipeptide antibodies were reacting with both gp120 and rabies virus glycoprotein.⁶ Analogous cross-reacting antibodies seem to be induced by vaccination with rabies virus, thus confirming the existence of a similar structural motif between the rabies virus glycoprotein and HIV-1 gp120. This common structural motif is probably related to a common functional feature that is binding to nicotinic receptors.

By using SPR, we have found that a component from detergent solubilized fetal calf membranes binds to HIV-1 gp120. The binding is inhibited by nicotine, which is a selective ligand of nicotinic acetylcholine receptor. Moreover, the binding is inhibited also by the synthetic peptide B2, which reproduces the putative nicotinic receptor binding motif of gp120.

These data, together with our previously described finding that gp120 is able to inhibit the binding of the snake neurotoxin α -bgt to nicotinic receptor in the human rhabdomyosarcoma cell line TE671,⁶ strongly indicate that HIV-1 gp120 can actually bind to muscle nicotinic receptors.

In conclusion, our results indicate that a region of HIV-1 gp120, such as rabies virus glycoprotein, can structurally and functionally mimic a snake neurotoxin specific receptor

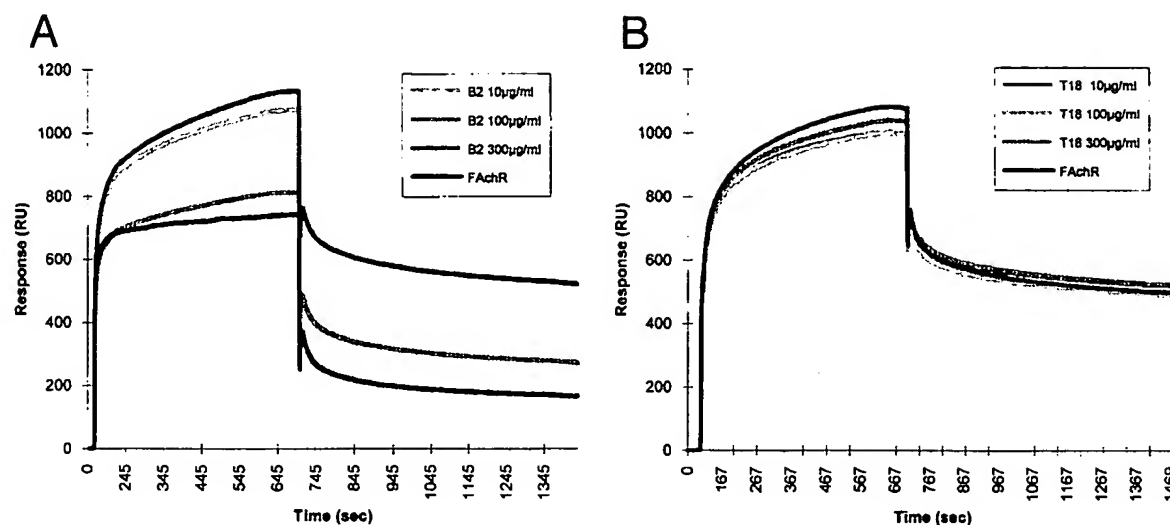


Fig 7. Inhibition of fetal calf muscle nicotinic receptor binding to HIV-1 gp120 by the B2 synthetic peptide. (A) Increasing amounts (10, 100, and 300 µg/mL) of the synthetic peptide B2 were mixed with the diluted detergent-solubilized receptor (0.5 nmol of ^{125}I - α -bgt bound/L). Aliquots of each mixture were injected at a flow rate of 5 µL/min in a flow cell where gp120 had been previously covalently immobilized. Regeneration of the matrix was obtained by injection of 10 mmol/L NaOH at the end of each cycle. (B) Identical concentrations of a peptide (T18) of the same length, but with a random sequence, were injected in identical experimental conditions.

binding motif. The ability of HIV-1 to bind to nicotinic receptors could be of primary importance in virus neuropathogenesis. Nicotinic receptors are widely distributed in the central nervous system.¹³⁻¹⁵ Even if the function of neuronal nicotinic receptor is not fully understood, some data indicate that they might be involved in the regulation of excitatory transmission in the central nervous system and in the release of transmitters such as glutamate^{16,17} and dopamine.¹⁸ Interestingly, stimulation of the $\alpha 7$ subunit of neuronal nicotinic receptors can induce an increase in presynaptic calcium influx, which is specifically blocked by α -bgt.^{16,17}

The neurotoxic effect of HIV-1 and gp120 has been reported in several studies¹⁹⁻²² despite the fact that neurons seem not to be infected *in vivo*.^{23,24} The binding of HIV-1 gp120 to nicotinic receptors in the brain could produce several dysfunctions in synaptic excitability, transmitters release, and calcium influx, even in the absence of the direct infection of neurones.

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